2011

Genetically Modified Es Cells Enhance Cardiac Repair And Regeneration In The Infarcted Heart

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GENETICALLY MODIFIED ES CELLS ENHANCE CARDIAC REPAIR AND REGENERATION IN THE INFARCTED HEART

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Burnett School of Biomedical Sciences
in the College of Graduate Studies
at the University of Central Florida
Orlando, Florida

Summer Term
2011

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ABSTRACT

Transplanted embryonic stem (ES) cells following myocardial infarction (MI) contribute to limited cardiac repair and regeneration with improved function. Therefore novel strategies are still needed to enhance the efficacy by which ES cells differentiate into cardiac cell types and inhibit adverse remodeling in the infarcted myocardium. Our studies evaluate whether genetic manipulation of transplanted ES cells employing miR-1, a pro-cardiac microRNA, and TIMP-1, an anti-apoptotic and anti-fibrotic protein, will enhance cardiac myocyte differentiation, inhibit native cardiac apoptosis, and reduce fibrosis in the infarcted myocardium. Furthermore, we assess levels of associated pro-(caspase-3, PTEN) and anti-(Akt) apoptotic proteins as well as a pro-fibrotic protein (MMP-9) in the post-MI and cell transplanted heart.

microRNAs (miRs) have emerged as critical regulators of various physiological processes including development, differentiation, metabolism, and death. Indeed, miR-1 plays an integral role in early cardiac development in *Drosophila* and mice as well as mediates differentiation of cardiac myocytes *in vitro*. To that end, we generated ES cells overexpressing miR-1 (miR-1-ES cells), transplanted them into the infarcted myocardium, and evaluated their impact on cardiac myocyte differentiation, myocardial repair, and left ventricular dysfunction post-MI. We provide evidence demonstrating enhanced cardiac myocyte commitment of transplanted miR-1-ES cells in the mouse infarcted heart as compared to ES cell and culture media transplanted hearts. Assessment of apoptosis revealed overexpression of miR-1 in transplanted ES cells protected host myocardium from MI-induced apoptosis through activation of p-Akt and inhibition of caspase-3, PTEN, and superoxide anion production. A significant reduction

in interstitial and vascular fibrosis was quantified in miR-1-ES and ES cell transplanted groups compared with control MI. However, no statistical significance between miR-1-ES cell and ES cell groups was observed. Finally mice receiving miR-1-ES cell transplantation post-MI had significantly improved heart function compared with respective controls. Our data suggests miR-1 drives cardiac myocyte differentiation from transplanted ES cells and inhibits apoptosis post-MI ultimately giving rise to enhanced cardiac repair, regeneration, and function.

Next, we assessed the role of miR-1-ES cells in a chronic model of MI as research has shown that apoptosis occurs not only hours but months following ischemia. 4 weeks following transplantation into the infarcted myocardium, we provide evidence demonstrating reduced cardiac apoptosis in miR-1-ES cell transplanted hearts compared to respective controls. Moreover, we show significant elevation of p-Akt levels and diminished PTEN levels in hearts transplanted with miR-1-ES cells as determined by enzyme-linked immunoassays. Finally, using echocardiography, we reveal mice receiving miR-1-ES cell transplantation post-MI had significantly improved cardiac function compared with animals transplanted with ES cell and culture media. Our data suggests that miR-1, when overexpressed in transplanted ES cells, has the capacity to inhibit apoptosis long term while attenuating contractility loss.

In addition to enhancing cardiac-specific donor cell differentiation, improving the efficacy by which stem cells promote cell survival and repair in the host myocardium is imperative in the pursuit of refining and optimizing stem cell therapy. To that end, we overexpressed TIMP-1, an endogenous inhibitor of apoptosis and fibrosis, in ES cells (TIMP-1-ES cells), transplanted them into infarcted myocardium, and evaluated their

impact on adverse cardiac remodeling. Immunofluorescence, TUNEL staining, caspase-3 activity, ELISAs, histology, and echocardiography were used to assess apoptosis, fibrosis, and heart function. Hearts transplanted with TIMP-1-ES cells demonstrated a reduction in apoptosis as well as an increase in p-Akt activity compared with ES cells or culture media controls. Interstitial and vascular fibrosis was significantly decreased in the TIMP-1-ES cell group compared to controls. Furthermore, MMP-9, a key pro-fibrotic protein, was significantly reduced following TIMP-1-ES cell transplantation. Echocardiography data showed fractional shortening and ejection fraction were significantly improved in the TIMP-1-ES cell group compared with respective controls. Our data suggest that transplanted ES cells overexpressing TIMP-1 attenuate adverse myocardial remodeling and improve cardiac function compared with ES cells.

Overall, our data suggest that genetic manipulation of ES cells following transplantation in the infarcted heart enhances cardiac myocyte differentiation, inhibits apoptosis and fibrosis as well as improves cardiac function.

To my children, Maddox and Kyla Fletcher, whose innocence and happiness have taught me the beauty of life and the patience to live it.

ACKNOWLEDGMENTS

“Around here, however, we don’t look backwards for very long. We keep moving forward, opening up new doors and doing new things… and curiosity keeps leading us down new paths.”
–Walt Disney

Although my name alone appears on this dissertation, a great many people have been instrumental in my success through their endless support and efforts. First and foremost, I would like to express my sincere respect and deepest gratitude to my advisor and mentor, Dr. Dinender Singla, for his valuable advice, extensive knowledge, generous support, and constructive guidance. I would like thank my committee members Dr. Steve Ebert, Dr. Jack Cheng, and Dr. Saleh Naser for their invaluable guidance, advice, and suggestions over the years. I would like to sincerely thank my fellow lab members for their continual support and encouragement during my professional and personal journeys throughout these past years. I would also like to thank the University of Central Florida, Burnett School of Biomedical Sciences, College of Medicine, for affording me access to their exemplary PhD program and associated facilities.

So many special thanks are owed my family for their unlimited love and support. To my husband, Rendon Fletcher, you have my deepest expression of love and appreciation for your patience, understanding, encouragement, and endless support. I wholeheartedly and sincerely give thanks to my mother, Grace Mario, who throughout life’s peaks and valleys, twists and turns, triumphs and sorrows, has been my source of strength and guidance and my model of faith, hope, and perseverance. I offer a very special thank you to my father, Kenneth Glass, whose unwavering faith and confidence

in my abilities have been the driving force behind my success. A thank you to my father, John Mario, whose willpower, drive, determination, and courage are a model to be followed by all. “A hero is an ordinary individual who finds the strength to persevere and endure in spite of overwhelming obstacles.” Christopher Reeve. I am eternally grateful to my mother-in-law, Yvonne O’Duaran, who in my absence during the rigorous hours of the program, has loved, nurtured, educated, and cared for my children. A simple thank you will never be enough. Finally, thank you to my brother and sisters for their continual support, encouraging words, and comic relief throughout my years of higher education.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFU	Arbitrary fluorescence units
Akt	Adenosine triphosphate-dependent tyrosine kinase
ANOVA	Analysis of variance
AU	Arbitrary units
Bcl2	B-cell lymphoma 2
BMP2	Bone morphogenetic protein-2
CC	Cell culture media
cDNA	Complementary deoxyribonucleic acid
Cdk9	Cyclin-dependent kinase-9
CM	Conditioned media
CMV	Cytomegalovirus
D14	Day 14
D28	Day 28
DAPI	4',6-diamidino-2-phenylindole
Dll-1	Notch ligand delta-like 1
DHE	Dihydroethidium
DMEM	Dulbecco’s modified eagle medium
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDV	Left ventricular volume at end diastole
EGF	Epidermal growth factor

EJ	Ejection fraction
ELISA	Enzyme-linked immunosorbent assay
ES	Embryonic stem
ESV	left ventricular volume at end systole
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FGF-4	Fibroblast growth factor-4
FS	Fractional shortening
G418	Geneticin
GFP	Green fluorescent protein
H_2O_2	Hydrogen peroxide
H9c2	Rat cardiomyoblasts
H&E	Hematoxylin and eosin
Hand2	Heart and neural crest derivatives expressed 2
HDAC4	Histone deacetylase 4
HRP	Horse radish peroxidase
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IL-6	Interleukin-6
iPS	Induced pluriopotent stem
LAD	Left anterior descending artery
LIF	Leukemia inhibitory factor
LNA	Locked nucleic acid

LVIDd	Left ventricular internal dimension-diastole
LVIDs	Left ventricular internal dimension-systole
MBA-1	Mouse bone marrow stromal cells
MC3T3-E1	Murine osteoblasts
Mef	Myocyte-specific enhancer-binding factor-2
MG-63	Human osteosarcoma cells
MHC	Myosin heavy chain
MI	Myocardial infarction
miR-1	MicroRNA-1
miR-1-ES cells	MicroRNA-1 overexpressing embryonic stem cells
miRS	MicroRNAs
MMP-9	Metalloproteinase-9
MMPs	Metalloproteinases
mRNA	Messenger ribonucleic acid
MyoD	Master regulator of muscle differentiation
NIH	National institute of health
$O_2^{\bullet-}$	Superoxide
$OH^{\bullet}$	Hydroxyl
$ONOO^-$	Peroxynitrite
p-Akt	Phosphorylated Akt
p-PTEN	Phosphorylated phosphatase and tensin homolog
PAN	Phospho-Akt1
PCR	Polymerase chain reaction

PI3K	Phosphoinositide-3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation
RNAs	Ribonucleic acids
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SEM	Standard error of the mean
snRNA	Small nuclear ribonucleic acid
TGF-β2	Transforming growth factor β-2
TIMPs	Tissue inhibitor of metalloproteinases
TIMP-1	Tissue inhibitor of metalloproteinase-1
TIMP-1-ES cells	TIMP-1 overexpressing embryonic stem cells
TNF-α	Tumor necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UT-7	Erythroid cells
UTR	Untranslated region
VA	Vessel area
VF	Vessel fibrosis
VEGF	Vascular endothelial growth factor

CHAPTER ONE: INTRODUCTION

Prevalence of MI

By the year 2030, the American Heart Association predicts 40.5% of the United States population will have 1 or more forms of cardiovascular disease (CVD)(19). Current estimations reveal CVD affects >81 million Americans with more than 10% afflicted by MI(19). MI events are becoming more prevalent, in part, due to the aging population, sedentary behavior, and unhealthy lifestyle habits (smoking, diet, etc). Recent advancements have been made in pharmaceutical interventions for the treatment of MI but long- and short-term morbidity and mortality remain significantly elevated(19). Additionally, heart transplantation has yet to become a feasible option due to the scarcity of organ donors. To that end, novel therapeutic strategies are needed to offer alternatives to the current treatments for MI.

Myocardial Infarction Pathophysiology

MI involves various intrinsic physiological and pathological changes to the myocardium. Due to prolonged ischemia following an MI event, cardiac myocyte cell death occurs(1). Cardiac cell death within the infarct and peri-infarct regions occurs as a result of apoptosis as well as necrosis(3; 15). Apoptosis, discrete from necrosis by varying morphological and biochemical changes, is a highly orchestrated programmed cell death characterized by cell blebbing and shrinkage, chromatin condensation, and DNA fragmentation. Apoptosis consequent to MI is commenced through various stressors including cytokines, oxidative stress, and DNA damage(1; 14; 15). Taking into consideration the inadequate regenerative capacity of the heart and the limited number

of resident cardiac progenitor stem cells, intrinsic mechanisms to replace lost cardiac myocytes following MI is ineffectual(24; 30). In response to cardiac myocyte cell loss within the infarcted myocardium, an increase in matrix metalloproteinases (MMPs) occurs leading to extracellular matrix (ECM) degradation, increased matrix protein production, fibrosis, stiffening of the heart, and cardiac dysfunction.

Stem Cells in the Infarcted Myocardium

Within the last decade, investigators have been exploring the potential use of cell-based therapies to attenuate cardiac cell damage and death and left ventricular dysfunction consequent to MI. A number of candidate cell types have been transplanted in the infarcted myocardium including skeletal myoblasts, bone marrow stem cells, bone marrow-derived hematopoietic stem cells, endogenous cardiac stem cells, mesenchymal stem cells, induced pluripotent stem (iPS) cells, and ES cells(13; 21-23; 26). Although the therapeutic efficacy of the various cell types used for transplantation studies have been inconsistent, ES cells possess many desirable traits including not only their plasticity but also their ability to influence injured myocardium through paracrine mechanisms making them a promising candidate cell type for use in post-MI cell transplantation(29).

ES cells, derived from the inner cell mass of the blastocyst, are pluripotent, undifferentiated cells. They are capable of self-renewal and are able to differentiate into cell types arising from all 3 germ layers. Noticeably, previous studies have demonstrated transplanted ES cells into the infarcted myocardium differentiate into all cardiac cell types including cardiac myocytes, endothelial cells, and vascular smooth

muscle cells(4; 26). Transplanted ES cells post-MI have further been shown to specifically migrate to the area of left ventricular injury, demonstrate engraftment, repair and regenerate the injured myocardium, and improve contractile function(27). In spite of previous assimilated knowledge, the efficacy by which ES cells differentiate into cardiac myocytes and inhibit adverse remodeling is limited(4; 6; 22; 27). To that end, current research is aimed towards genetic manipulation of ES cells to optimize the therapeutic potential of these cells for cell transplantation in the infarcted myocardium.

microRNA-1 in Cardiac Myocyte Differentiation

Cardiac myocyte commitment from transplanted stem cells in the infarcted myocardium remains a major challenge to post-MI cell therapy. Previous reports have concluded only ~ 5-15% of transplanted stem cells survive and demonstrate engraftment compared to the remaining 85-95% of transplanted cells which undergo cell death due to apoptosis or necrosis(6; 25; 37). Of those 5-15% cells which engrafted, limited cardiac myocyte differentiation was observed within this cell population (6; 22). A multitude of attempts have been made to enhance and drive cardiac myocyte differentiation from stem cells using various factors including bone morphogenetic protein-2 (BMP2), transforming growth factor β-2 (TGFβ–2) , vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and -2 (IGF-2), activin-A, fibroblast growth factor-2 (FGF-2) and -4 (FGF-4), interleukin-6 (IL-6), and epidermal growth factor (EGF)(2; 31; 35; 36). Despite these efforts, the observed donor cell differentiation into cardiac myocytes remains insufficient to regenerate adequate *de novo* myocardium to restore the injured heart to pre-MI homeostasis(6; 22)

microRNAs (miRs) are small, single-stranded, non-coding RNAs which recognize the 3' untranslated region (UTR) of their target mRNA and modulate gene expression through mRNA degradation, translational repression, and deadenylation. Recently, miRs have been identified in the regulation of diverse physiological and pathological cellular processes including development, differentiation, proliferation, metabolism, and death(7; 12; 16; 20). Increasing evidence illustrating the ability of miRs to govern complex biological processes underscores the potential of genetic manipulation imploring miRs for therapeutic applications.

miR-1, a widely conserved muscle- and cardiac-specific microRNA, has been identified as a major regulator of cardiac muscle commitment and differentiation(16; 38; 39). Evidence has shown that overexpression of miR-1 in fibroblasts altered the cell gene expression towards that of a muscle cell and when overexpressed in C2C12 myoblasts, miR-1 induced myogenic differentiation as well as associated cardiac markers including myosin heavy chain (MHC), myogenin, MyoD, Mef2, and α-actin(5; 18). Furthermore, *in vitro* studies exploiting mouse and human ES cells and cardiac-derived progenitor cells revealed increased miR-1 expression upon cardiac differentiation induction(11; 32; 33). However, whether miR-1 overexpression in transplanted ES cells will drive cardiac myocyte differentiation in the injured myocardium is unknown.

TIMP-1 in Cardiac Remodeling

In addition to enhancing cardiac-specific donor cell differentiation, improving the efficacy by which stem cells promote host myocardium cell survival and repair is

imperative in the pursuit of refining and optimizing stem cell therapy. Following MI and myocardial cell loss, cardiac remodeling commences in response to increased MMP expression. MMPs are a family of endopeptidases which play a pivotal role in the degradation and aberrant production of ECM proteins leading to adverse alterations in ventricular architect post-MI. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous protease inhibitors of MMPs. TIMPs non-covalently bind the active site of inert MMP pro-forms, blocking substrate access and activation of MMPs. TIMP-1 not only exhibits anti-fibrotic characteristics, but recently has been shown to be anti-apoptotic in non-cardiac cell lines(8-10; 17; 28; 34). Noticeably, we recently reported TIMP-1 to be a cytoprotective released factor from ES cells preventing H_2O_2 induced apoptosis in H9c2 cardiomyoblasts(28). However, the effects of transplanted ES cells overexpressing TIMP-1 on host myocardium remodeling post-MI remains obscure.

Rationale and Aims

MI is a leading cause of death in the western hemisphere consequent to myocardial injury (cardiac myocyte cell death and fibrotic scar formation) sustained during prolonged ischemia(3; 19; 25). The intrinsic regenerative mechanisms of the heart are unable to replenish the infarcted myocardium with significant amounts of functional cardiac myocytes to compensate for the millions of cells lost during an MI event(1; 14). Additionally, conventional medical options have not been wholly successful in catapulting the heart back to pre-MI status. Recently, exhaustive effort has been put into the development of cell transplantation therapy for treatment of MI as an alternative method to repopulate the infarcted area with functional cardiac myocytes

thus regenerating the apoptotic myocardium and rescuing contractile function. Previous studies have shown transplanted ES cells in the infarcted myocardium engraft, differentiate into cardiac cells, and inhibit adverse left ventricular remodeling (apoptosis, fibrosis, and hypertrophy)(26; 27). However, the efficacy by which transplanted ES cells differentiate into cardiac myocytes and inhibit adverse remodeling is limited. Therefore current research in stem cell transplantation is now focused on optimization and refinement of ES cell populations using genetic manipulation.

Within the presented research, we generated ES cells overexpressing miR-1, a pro-cardiac microRNA, transplanted them into the infarcted myocardium, and evaluated their effects on cardiac myocyte commitment, adverse remodeling, and left ventricular function in the acute setting of MI. The goal of the first study was to enhance cardiac myocyte differentiation from transplanted ES cells post-MI. We hypothesized that miR-1-ES cells would more readily differentiate into cardiac myocytes compared with untransfected ES cells following transplantation into the infarcted myocardium. To test our hypothesis, the following aims were proposed:

1. Determine the effects of miR-1 overexpression in ES cells on cardiac myocyte differentiation following transplantation in the infarcted myocardium
2. Assess the impact of miR-1-ES cells on host myocardium apoptosis following MI
3. Identify mechanisms of apoptosis inhibition following ES cell transplantation
4. Resolve the impact of transplanted miR-1-ES cells on left ventricular function following MI

Within the second study, we evaluated the impact of miR-1 overexpression in transplanted ES cells on apoptosis and function in the chronic model of MI. The goal of

this study was to demonstrate long term beneficial effects of miR-1 overexpression in transplanted ES cells. We hypothesized that miR-1-ES cells would significantly inhibit apoptosis and improve cardiac function 4 weeks following transplantation into the infarcted myocardium compared to unmodified ES cells. To test our hypothesis, the following aims were proposed:

1. Evaluate the impact of transplanted miR-1-ES cells on host myocardium apoptosis 4 weeks following transplantation
2. Elucidate mechanisms of miR-1-ES cell-mediated apoptotic inhibition
3. Determine the impact of transplanted ES cells overexpressing miR-1 on cardiac function 4 weeks post-MI

Finally, within a third study, we generated ES cells overexpressing TIMP-1, an anti-apoptotic and anti-fibrotic protein, transplanted them into the infarcted myocardium, and evaluated their impact on apoptosis, fibrosis, and associated cardiac function in an acute setting of MI. The goal of the second study was to optimize the efficacy by which ES cells inhibit apoptosis and fibrosis and ultimately left ventricular dysfunction. We hypothesized TIMP-1-ES cells following transplantation into the infarcted myocardium would attenuate apoptosis and fibrosis along with improve cardiac function. To test our hypothesis, we proposed the following aims:

1. Assess the efficacy by which transplanted TIMP-1-ES cells inhibit host myocardium apoptosis
2. Identify mechanisms by which TIMP-1-ES cells mediate apoptotic inhibition
3. Determine the impact of transplanted TIMP-1-ES cells on cardiac fibrosis following MI

4. Evaluate the effect of TIMP-1-ES cells on cardiac dysfunction post-MI

The long term goal of our research is to generate an ideal stem cell population for cardiac repair and regeneration following MI which readily differentiates into cardiac myocytes, effectively inhibits apoptosis and fibrosis, and significantly restores cardiac function. Generation of such a stem cell population would provide a strong foundation to pursue future research transcending these optimized ES cells from the bench to the clinical arena.

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CHAPTER TWO: microRNA-1 TRANSFECTED EMBRYONIC STEM CELLS ENHANCE CARDIAC MYOCYTE DIFFERENTATION AND INHIBIT APOPTOSIS BY MODULATING PTEN/AKT PATHYWAY IN THE INFARCTED HEART

Abstract

microRNAs (miRs) have emerged as critical modulators of various physiological processes including stem cell differentiation. Indeed, miR-1 has been reported to play an integral role in the regulation of cardiac muscle progenitor cell differentiation. However, whether overexpression of miR-1 in embryonic stem (ES) cells (miR-1-ES cells) will enhance cardiac myocyte differentiation following transplantation into the infarcted myocardium is unknown. In the present study, myocardial infarction (MI) was produced in C57BL/6 mice by left anterior descending artery (LAD) ligation. miR-1-ES cells, ES cells, or culture medium (CC, control) was transplanted into the border zone of the infarcted heart and 2 weeks post-MI, cardiac myocyte differentiation, adverse ventricular remodeling, and cardiac function were assessed. We provide evidence demonstrating enhanced cardiac myocyte commitment of transplanted miR-1-ES cells in the mouse infarcted heart as compared to ES cells. Assessment of apoptosis revealed overexpression of miR-1 in transplanted ES cells protected host myocardium from MI-induced apoptosis through activation of p-AKT and inhibition of caspase-3, PTEN, and superoxide production. A significant reduction in interstitial and vascular fibrosis was quantified in miR-1-ES cell and ES cell transplanted groups compared with control MI. However, no statistical significance between mir-1-ES cell and ES cell groups was observed. Finally, mice receiving miR-1-ES cell transplantation post-MI had significantly improved heart function compared with respective controls ($p<0.05$). Our data suggest miR-1 drives cardiac myocyte differentiation from transplanted ES cells

and inhibits apoptosis post-MI ultimately giving rise to enhanced cardiac repair, regeneration, and function.

Introduction

Following myocardial infarction (MI), various intrinsic pathological processes occur within the myocardium including oxidative stress-induced apoptosis giving rise to stiffening of heart muscle, impaired cardiac function, and, ultimately, heart failure and death(3; 17; 18; 22; 29). To date, treatment for MI with pharmaceutical agents have yet to be wholly successful and due to the scarcity of organ donors, heart transplantation has yet to be a feasible option. Given this, stem cell therapy has gained considerable attention as an alternate method to repair or regenerate injured myocardium consequent to MI. Various investigations have demonstrated the ability of stem cells transplanted into the infarcted heart to engraft, differentiate into cardiac cell types, inhibit apoptosis and fibrosis, and improve cardiac function(4; 16; 28; 29). However, previous reports have revealed transplanted stem cells into the infarcted myocardium demonstrate limited cardiac myocyte differentiation(8; 23). Therefore, innovative stratagem aimed at enhancing cardiac myocyte differentiation from transplanted stem cells is monumental in the pursuit of optimizing stem cell transplantation for post-MI therapy.

Recently, microRNAs (miRs) have been identified as regulators of various physiological and pathological heart processes including cardiac development, differentiation, arrhythmias, hypertrophy, remodeling, and angiogenesis(5; 6; 9; 10; 24; 36; 44). miRs are small, single stranded, non-coding RNAs usually about 20-24

nucleotides in length. They recognize the 3' untranslated region (UTR) of their target mRNA and negatively regulate gene expression through translational repression, mRNA degradation, and deadenylation. Genetic manipulation imploring miRs for therapeutic applications has recently gained significant interest in the research community but remains in its infancy.

miR-1, a widely conserved, cardiac- and muscle-specific miR, has been identified in early heart development (19; 46; 47). Previous studies have illustrated a relationship between miR-1 expression and myocardial differentiation through regulation of various cardiac markers including myosin heavy chain (MHC), myogenin, Hand2, MyoD, Mef2, Nkx2.5, Cdk9, and α-actin(7; 36; 47). However, exploitation of miR-1 in ES cells for post-MI therapy has yet to be attempted. Based on our previous findings and others, we hypothesize that overexpression of miR-1 in transplanted ES cells following MI will enhance cardiac myocyte differentiation from donor ES cells as well as attenuate MI-associated myocardial remodeling. In the present study, our data demonstrates that hearts transplanted with miR-1-ES cells contain significantly more donor cell-derived cardiac myocytes compared to respective controls. Additionally, we reveal that transplanted miR-1-ES cells significantly inhibit apoptosis through modulation of p-Akt and PTEN gene expression. Moreover, we show miR-1-ES cells attenuate oxidative stress in the injured myocardium. Finally, our data demonstrate enhanced cardiac function following transplantation of miR-1-ES cells post-MI. Our study establishes a strong foundation and rationale to pursue future research using microRNAs in the ongoing development of ES cell transplantation therapy for the treatment of myocardial infarction.

Materials and Methods

ES Cell Culture

Mouse CGR8 ES cells were passaged and maintained as previously reported (33). In brief, cells were plated on 0.1% gelatin-coated tissue culture dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% ES-qualified FBS, leukemia inhibitory factor (LIF), glutamine, non-essential amino acids, penicillin/streptomycin, β-mercaptoethanol, and sodium pyruvate.

miR-1-ES Cell Generation

Mouse pre-miR-1 oligonucleotides (5'-tgc tgT GGA ATG TAA AGA AGT ATG Tag ttt tgg cca ctg act gac TAC ATA CTT TTA CAT TCC A-3' and 5'-cct gTG GAA TGT AAA AGT ATG Tag tca gtc agt ggc caa aac TAC ATA CTT CTT TAC ATT CCA c-3') were cloned into a pcDNA 6.2-GW/EmGFP expression vector (Invitrogen) containing the selectable marker blasticidin. The expression of miR-1 and GFP was driven by a cytomegalovirus (CMV) promoter. CGR8 mouse ES cells were transfected with the pre-miR-1 expressing vectors using Lipofectamine 2000 (Invitrogen). Selection media containing 2 μg/ml blasticidin (Invitrogen) was added 48 hrs post-transfection and was changed every 48 hrs. 2-3 weeks thereafter, blasticidin resistant ES cell colonies (labeled miR-1-ES cells) were selected, expanded, and maintained in ES cell culture medium. For control cells, a subset of CGR8 ES cells was transfected with the mammalian expression vector pTurboFP635-C (Evrogen) encoding RFP.

RNA Extraction and Real-time RT-PCR

Total RNA was isolated from ES and miR-1-ES cells using RNA STAT-60 (Tel-Test) and reverse transcribed using Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time RT-PCR was performed with specific miR-1 primers (Assay ID# 002222, Applied Biosystems) and Taqman® Universal PCR Master Mix (Applied Biosystems) using a CFX96™ Real-Time System (Bio Rad). Gene expression was normalized to U6 snRNA (Assay ID# 001973, Applied Biosystems). Data was analyzed and the miR-1 fold change calculated using the Bio Rad CFX Manager software (Version 1.6).

MI and ES Cell Transplantation

All mice were maintained and used as approved by the University of Central Florida animal review board. 8-10 week old C57BL/6 mice (Jackson laboratories) were divided into 4 study groups (n=8): Sham, MI + cell culture media (MI + CC), MI + ES cells, and MI + miR-1-ES cells. In brief, mice were anesthetized with 2.5% isoflurane, intubated, and ventilated using a rodent MiniVent (Harvard Apparatus). Following a left thoracotomy, the left anterior descending (LAD) coronary artery was visualized and a 7-0 ligature (CP Medical) was placed around the coronary artery. Following LAD ligation, two separate intramyocardial injections of 10μl of media with or without 2.5×10^4 cells were delivered into the peri-infarct region. The ribs, muscle, and skin were sutured, lungs expanded, and mice extubated following weaning from the ventilator. Sham animals received all surgical procedures as detailed above excluding the LAD ligation. At D14 following surgery, mice were sacrificed with pentobarbital (80 mg/kg) followed by

cervical dislocation. Hearts were removed, transversely cut, and fixed in formalin for further assessment.

Immunohistochemistry

Heart sections were deparaffinized and immunohistochemically stained using primary antibodies against RFP (for ES cells; AB232, Evrogen) or GFP (for miR-1-ES cells; A3122, Invitrogen) and sarcomeric α-actin (A2172, Santa Cruz). Heart sections were then incubated with Alexa 488- or Alexa 568- conjugated secondary antibodies (Invitrogen), respectively. Heart sections were washed and mounted with Anti-fade Vectashield mounting medium as mentioned above. Co-localization of α–actin positive cells with GFP or RFP cells were counted as newly differentiated cells from transplanted donor cells in 1-2 sections from n=5-8 animals/group. Sections were examined with Olympus and Leica TSC SP2 laser scanning confocal microscopes.

Histopathology

In brief, heart tissue was fixed in 4% buffered formalin, embedded in paraffin, and cut into 5 μm serial sections. Embedded paraffin heart sections were placed onto Colorfrost Plus microscope slides (Fisher Scientific), deparaffinized, and rehydrated as previously reported(29). Thereafter, heart sections were stained with either hematoxylin and eosin (H&E) for teratoma evaluation or Mason's trichrome for visualization of interstitial and vascular fibrosis. Using NIH Image J software, interstitial fibrotic area was calculated by measuring the collagen-positive blue area (mm^2) within the infarct, peri-infarct and non-infarct regions in 1-2 sections from 5-8 hearts/group. Vascular

fibrosis was calculated as the ratio of vascular fibrosis to vessel area x 100% in 1-2 sections from 5-8 hearts/group.

TUNEL Staining

TUNEL staining and analysis was performed as previously described by us and others(12; 15; 31; 32). In brief, heart sections were deparaffinized and permeabilized with proteinase K (25 µg/ml in 100 mM Tris·HCl). An in situ apoptotic cell death detection kit (TMR red; Roche Applied Biosystems) was used to detect host myocardium apoptotic nuclei. Heart sections were thereafter mounted with Anti-fade Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) for nuclear visualization. Sections observed under Olympus and confocal microscopes. The percentage of total apoptotic nuclei was determined in the infarct and peri-infarct regions from n=5-8 animals/group. Percentage total apoptotic nuclei = (total number apoptotic nuclei) / (total number of DAPI) x 100%. Heart sections were also stained with an anti-sarcomeric-α-actin antibody (A2172, Santa Cruz) to differentiate between apoptosis in cardiac myocytes and non-cardiac myocytes. The percentage of cardiac myocyte apoptotic nuclei was calculated by (total number of red stained apoptotic nuclei co-localized with sarcomeric α-actin) / (total number of blue stained nuclei with DAPI) x 100%.

Caspase-3 Activity Assay

Caspase-3 activity assay was performed using a caspase-3 colorimetric activity assay kit from BioVision (K106-200). Heart tissue was homogenized in radioimmunoprecipitation (RIPA) buffer containing protease inhibitors,

phenylmethylsulfonyl fluoride (PMSF), sodium orthovandate, and sodium fluoride. Homogenized heart tissue was centrifuged at 14,000 g for 5 min. Supernatant was removed and placed into fresh centrifuge tubes. Protein concentration was estimated using a Bio Rad assay. Caspase-3 activity was performed per the manufacturer's instructions and color reaction was measured in a microtiter plate reader (Bio Rad) at 405 nm. Capsase-3 activity was plotted as arbitrary units (A.U.). Data was collected from heart homogenates of n=6-8 animals/group in duplicates.

DHE Staining

Superoxide generation within infarcted myocardium was determined using dihydroethidium (DHE) fluorescence staining. Following deparaffinization and hydration, heart sections were stained with 1μM/mL DHE for 25 minutes at room temperature. The fluorescent images were captured on an Olympus and confocal microscope. Using Image J, DHE fluorescence intensity was quantified in 4-5 randomly selected regions in the peri-infarct zone/section in 7-8 animals/group as previously reported (25). The average corrected integrated density (after background correction) was calculated for each group and DHE fluorescence intensity was plotted as arbitrary fluorescence units (A.F.U.).

Akt Activity Assay

p-Akt was quantitated using a phospho-Akt1 (PAN) ELISA kit (X1844k, Exalpha Biological) as detailed in the provided protocol. In brief, hearts were homogenized and proteins were estimated as described above. Samples were added to the wells containing captured antibodies provided in the kit and incubated with detector antibody,

HRP conjugate, substrate, and a stop solution with washings in between each step. The developed color reaction was measured in a microtiter plate reader (Bio Rad) at 450 nm. The values obtained from the ELISA were normalized to the total protein concentration for each sample as determined by the Bradford assay. Phospho-Akt graph was plotted as arbitrary units. Data was collected from heart homogenates of n=6-8 animals/group in duplicates.

PTEN ELISA

Phospho-PTEN (p-PTEN) was quantified using a Cell Signaling Technology® Phospho-PTEN Sandwich ELISA kit (#7285) as detailed in the instruction manual. The developed reaction, proportional to the quantity of p-PTEN, was measured at 450 nm in a microtiter plate reader (Bio Rad). ELISA results were corrected for the protein concentration of each tissue sample. p-PTEN data was collected from heart homogenates from n=6-8 animals/group in duplicates and plotted as arbitrary units.

MMP-9 Immunoassay

MMP-9 concentration was determined by an enzyme-linked immunoassay (MMPT90, R&D Systems) as described by others and in the protocol provided with the kit(35; 40). A color reaction, proportional to the amount of bound MMP-9, was measured in a microtiter plate reader (Bio Rad) at 450 nm. Based on protein content of each sample, determined by Bradford assay, ELISA results were corrected. MMP-9 data was collected from heart homogenates from n=5-8 animals/group in duplicates and plotted as arbitrary units.

Echocardiography

Cardiac function was assessed 2 wks post-MI by transthoracic echocardiography. Mice were anesthetized with 2% inhalant isoflurane using a nose cone system and placed on a temperature controlled heating pad. Echocardiography was performed using a Sonos 5500 Ultrasound system with a 15-6L hockey stick transducer. Left ventricular internal dimension-diastole, left ventricular internal dimension-systole, fractional shortening (LVIDd-LVIDs/LVIDd x 100), and ejection fraction ((left ventricular volume at end diastole (EDV) – left ventricular volume at end systole (ESV))/EDV, EDV and ESV were calculated using the Teichholz formula(38)) were assessed in short axis view at the mid-papillary muscle level.

Statistical Analysis

All values presented as a mean ± standard error of the mean (SEM) and were analyzed using SigmaStat3.5 software. Analysis of data was performed using the students' t-test, one-way analysis of variance (ANOVA), and the Bonferroni test. Statistical significance was accepted for $p<0.05$.

Results

Figure 1A, a schematic representation showing pre-miR-1 oligonucleotide sequence and vector design used to overexpress miR-1 in ES cells (miR-1-ES cells). Following transfection, cells were selected and maintained in routine cell culture for 2-3 wks. RT-PCR performed on blasticidin-resistant colonies demonstrate more than a seven-fold increase in miR-1 expression in miR-1-ES cells compared to parental ES

cells (Figure 1B). Additionally, Figure 1C (phase contrast) and 1D (green fluorescence) photomicrographs depict successful generation of miR-1-ES cells expressing GFP.

miR-1 Enhances Cardiac Myocyte Differentiation

To assess whether miR-1 drives differentiation of transplanted ES cells into cardiac myocytes post-MI, heart sections were double immunolabeled for the cardiac myocyte marker α-actin and for GFP (miR-1-ES cells) or RFP (ES cells) to detect donor differentiated cells. Co-localization of α-actin-positive cells with GFP or RFP positive cells were identified and counted as newly differentiated cardiac myocytes from donor ES cells. Figures 2A-C show α-actin positive cardiac myocytes (red), 2D-F show transplanted donor cells (green), 2G-I show total nuclei stained with DAPI (blue), and 2J-L show merged images of triple-labeled sections depicting cardiac myocyte differentiated from donor transplanted cells. miR-1-ES cell transplanted hearts contained significantly more donor differentiated cells compared to ES cell and cell culture media transplanted hearts (mean ± SEM; MI + miR-1-ES cells: 38.33 ± 4.61 vs. MI + ES cells: 17.12 ± 2.23 and MI + CC: 0.00 ± 0.00, $p<0.001$, Figure 2E) suggesting miR-1 plays a major role in the differentiation of transplanted ES cells into cardiac myocytes post-MI.

Transplanted miR-1-ES Cells Prevent Apoptosis in the Infarcted Heart

TUNEL staining was performed and representative photomicrographs are depicted in Figure 3A-L. At D14, there was a significant decrease in TUNEL-positive nuclei post-MI in miR-1 ES cell transplanted groups compared to ES cell and medium

groups (mean ± SEM; MI + miR-1 ES cells: 0.55 ± 0.06% vs. MI + ES cells: 0.87 ± 0.05% and MI + CC: 1.45 ± 0.14% TUNEL positive nuclei/total nuclei, respectively, $p<0.05$, Figure 3Q). Next, apoptosis in cardiac myocytes post-MI was determined in heart sections by co-labeling with sarcomeric α-actin (Figure 3M-P). Our data reveals miR-1-ES cell transplanted hearts contained significantly fewer apoptotic cardiac myocyte nuclei compared to respective controls ($p<0.05$, Figure 3R). To reinforce our TUNEL data, a caspase-3 activity assay was performed on heart homogenates from 5-8 animals/group. A significant decrease in caspase-3 activity in hearts transplanted with miR-1-ES cells post-MI compared to hearts transplanted with ES cells or medium alone resulted ($p<0.05$, Figure 3S).

miR-1 Enhances p-Akt Activation in Post-MI Hearts

Previous studies have identified direct and indirect mechanisms of apoptotic inhibition through activation of various cell survival cascades such as adenosine triphosphate-dependent tyrosine kinase (Akt)(33). Using a phospho-Akt ELISA, we determined that hearts transplanted with miR-1-ES cells had significant activation of p-Akt compared to hearts transplanted with ES cells and cell culture medium (mean ± SEM; MI + miR-1-ES cells: 6.88 ± 0.29, vs. MI + ES cells: 5.45 ± 0.14 and MI + CC 3.86 ± 0.59, $p<0.05$, Figure 4A). To evaluate whether increased activation of p-Akt was attributable to inhibition of phosphoinositide-3-kinase (PI3K)/Akt signaling, levels of phosphatase and tensin homolog (PTEN), an inhibitor of PI3K phosphorylation, were quantified. Our data illustrates a significant reduction in PTEN concentration in hearts transplanted with miR-1-ES cells vs. hearts transplanted with ES cells or cell culture

medium (mean ± SEM; MI + miR-1-ES cells: 6.49 ± 0.20, vs. MI + ES cells: 8.12 ± 0.44 and MI + CC 10.08 ± 0.47, $p<0.05$, Figure 4B).

Transplanted miR-1-ES Cells Reduce MI-Induced Oxidative Stress

To examine the consequence of miR-1-ES cells on MI-induced oxidative stress, we analyzed intracellular superoxide anion production in heart sections using DHE fluorescent staining at D14 following MI or sham surgery. As shown in the representative photomicrographs (Figure 5A-L) and summary data (Figure 5M), DHE staining was significantly reduced in miR-1-ES cell transplanted hearts compared with ES cell and cell culture medium transplanted hearts (mean ± SEM; MI + miR-1-ES cells: 3.00 ± 0.53, vs. MI + ES cells: 6.08 ± 0.43 and MI + CC 11.37 ± 1.20, $p<0.05$, Figure 5M).

Effects of miR-1-ES Cells on Interstitial and Vascular Fibrosis

Heart sections were stained with Mason's trichrome and interstitial fibrosis (Figure 6A-D) was quantified. Quantitative analysis of trichrome stained heart sections indicated, compared to medium alone, both miR-1-ES cell and ES cell groups had a significant reduction in the amount of interstitial fibrosis (mean ± SEM; MI +CC: 1.02 ± 0.06 mm^2 vs. MI + miR-1-ES =cells: 0.36 ± 0.04 mm^2 and MI + ES cells: 0.49 ± 0.03 mm^2, $p<0.05$, Figure 6I). However, there was no statistical significance in interstitial fibrosis between miR-1-ES cell and ES cell groups. Next, quantitative analysis of vascular fibrosis (Figure 6E-H) revealed miR-1-ES cell and ES cell transplanted hearts had a significant reduction in the amount of vascular fibrosis compared to hearts transplanted with cell culture medium ($p<0.001$, Figure 6J). Again, as with interstitial

fibrosis, statistical significance was not reached between miR-1-ES cell and ES cell groups for vascular fibrosis.

Furthermore, matrix metalloproteinase-9 (MMP-9), a mediator of extracellular matrix (ECM) degradation and indicator of myocardial fibrosis post-MI, was quantified. miR-1-ES cell and ES cell transplanted hearts demonstrated significantly reduced MMP-9 concentration compared with control hearts ($p<0.01$, Figure 6K). However, as observed with interstitial and vascular fibrosis, there was no significant difference between miR-1-ES cell and ES cell groups.

No Teratoma Formation in miR-1-ES Cell Transplanted Hearts

ES cells have the capacity to differentiate into all derivatives of ectoderm, endoderm, and mesoderm, presenting the risk of teratoma formation at the injection site. However, upon inspection of whole hearts and H&E stained heart sections, no teratoma formation was observed in any of the miR-1-ES cell or ES cell transplanted hearts (data not shown).

Overexpression of miR-1 Improves Cardiac Function Post-MI

Echocardiography data showed mice receiving ES cell transplantation following MI had significantly improved fractional shortening compared to medium alone ($p<0.001$, Figure 7A). Additionally, mice transplanted with miR-1-ES cells post-MI had significantly increased fractional shortening when compared to both ES cell and medium control groups (mean ± SEM; MI + miR-1-ES cells: 43.88 ± 0.33%, vs. MI + ES cells: 40.53 ± 0.74% and MI + CC 32.33% ± 0.97%, $p<0.01$, Figure 7A). Furthermore, miR-1-

ES cell transplanted mice post-MI also had significantly improved ejection fraction when compared with mice transplanted with ES cells or medium post-MI ($p<0.05$, Figure 7B).

Discussion

The efficacy of cardiac myocyte differentiation from transplanted ES cells remains a major challenge to post-MI cell therapy. Numerous attempts have been made to enhance and drive cardiac myocyte differentiation from stem cells using various growth factors including bone morphogenetic protein-2 (BMP2), transforming growth factor β-2 (TGFβ−2) , vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and -2 (IGF-2), activin-A, fibroblast growth factor-2 (FGF-2) and -4 (FGF-4), interleukin-6 (IL-6), and epidermal growth factor (EGF)(2; 34; 42; 45). Despite these efforts, the observed donor cell differentiation into cardiac myocytes remains insufficient to regenerate the infarcted heart(8; 23). Recently, miRs have been implicated in the differentiation of a myriad of cells types including epidermal cells, adipocytes, osteoblasts, neuronal cells, and cardiac muscle(11; 19-21; 43). In the present study, we transfected ES cells with miR-1, a pro-cardiac microRNA, transplanted them into the myocardium post-MI, and evaluated their effects on cardiac myocyte differentiation, apoptosis, fibrosis, oxidative stress, and cardiac function.

Our data for the first time suggest miR-1, when transfected in ES cells, following transplantation into the infarcted myocardium, significantly increases the amount of donor cell-derived differentiated cardiac myocytes compared with untransfected ES cells. Our data corroborates with reports that miR-1 participates in early cardiac development in *Drosophila* and mice as well as enhances cardiac myocyte

differentiation in the cell culture system(19; 46; 47). Although several miR-1 targets have been identified in the developing heart and cell culture system including histone deacetylase 4 (HDAC4), heart and neural crest derivatives-expressed protein 2 (Hand2) and cyclin-dependent kinase-9 (Cdk9)(6; 36; 39), the mechanisms by which miR-1 transfected ES cells promote cardiac differentiation in the infarcted heart remains unknown.

Next, it was imperative to investigate whether transplanted miR-1-ES cells promote additional repair mechanisms via inhibition of apoptosis and fibrosis in the infarcted myocardium. In the current study, we report that transplanted miR-1-ES cells significantly reduce endogenous cardiac myocyte apoptotic cell death in the infarcted heart compared with untransfected ES cells. Our data is the first report demonstrating inhibition of cardiac myocyte cell death post-MI with transfected miR-1-ES cells. In contrast, several published reports have suggested that miR-1 is pro-apoptotic in cardiac myocytes following MI(26; 37). However, the experimental design reported in the current study is completely distinct compared to the studies in which miR-1 is reported to be apoptotic which may explain the discrepancies between the varying results(26; 37). Moreover, we are not the first group to report conflicting apoptotic data relative to miRs in various cell lines. In fact, Wang recently concluded upon reviewing published data on miR-21 that a single miR has the potential to be apoptotic, anti-apoptotic, or neutral depending on the cell line in which it is being expressed(41).

In an attempt to elucidate mechanisms by which miR-1-ES cells inhibit host myocardium apoptosis post-MI, we investigated the connection between miR-1 and the PI3K/Akt pathway. The PI3K/Akt pathway is a well-documented signaling cascade

which controls regulation of various cellular processes including cell growth, proliferation, and survival. Additionally, previous studies have demonstrated Akt to be cardioprotective and anti-apoptotic post-MI(13). To this end, we assessed the levels of activated Akt and were able to elucidate a positive correlation between Akt activation and apoptosis inhibition in miR-1-ES cell transplanted hearts. Specifically, we noted that as apoptosis was significantly decreased in miR-1-ES cell transplanted hearts, levels of p-Akt were significantly increased compared to ES cell and cell culture medium transplanted hearts. Although we were able to make this distinction, Akt mRNA is not a predicted target of miR-1. To that end, we turned our investigation to upstream components of the Akt pathway.

PTEN, an inhibitor of PI3K phosphorylation, blocks the subsequent activation and phosphorylation of Akt. Therefore we could circumvent that down regulation of PTEN in the heart would result in increased PI3K/Akt signaling leading to restrained cardiac myocyte apoptosis and inhibited maladaptive myocardial remodeling. Our data was in corroboration with this line of thought as PTEN levels were significantly decreased in hearts transplanted with miR-1-ES cells compared to ES cell or cell culture medium transplanted hearts. Although miR-1 is not predicted to target PTEN directly, negative regulation of PTEN activators may explain the increase in Akt activity and decrease in PTEN observed within this study. However, because predicted targets of miR-1 span a myriad of mRNAs with a host of biological activities, determination of whether miR-1 regulates these targets is beyond the scope of this study and requires further investigation.

Oxidative stress is a key mediator of myocardial apoptosis following MI(1; 30; 33). Oxidative stress-induced cardiac myocyte apoptosis involves the activation of 2 independent pathways (Bcl-2 family activated mitochondrial intrinsic pathway and Fas ligand or TNF-α extrinsic pathway) through generated reactive oxygen species (ROS) including superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$)(14). Because miR-1 is not limited to single target regulation, we examined whether miR-1-ES cells also confer direct anti-apoptotic protective effects on infarcted myocardium through reactive oxygen species (ROS) attenuation. Our data demonstrates that while MI induced the generation of superoxide in the myocardium, transplanted miR-1-ES cells blunted superoxide production. Our study suggests that miR-1-ES cells may prevent apoptosis not only through activation of the cell-survival PI3K/Akt pathway, but also through regulation of ROS generation.

In other respects, not only does adverse cardiac remodeling involve apoptosis but also the formation of fibrosis to rescue the architecture of the infarcted myocardium. Fibrosis formation involves the activation of metalloproteinases (MMPs), degradation of the extracellular matrix (ECM), and increased collagen deposition. Previous studies have shown that stem cells following transplantation post-MI inhibit fibrosis formation(27; 29). Although a trend of attenuated interstitial and vascular fibrosis was reported, our data shows that hearts transplanted with miR-1-ES cells post-MI demonstrate no significant additional decrease in fibrosis compared with untransfected ES cells. MMP-9, a well-established mediator of ventricular remodeling, was also assessed to establish a relationship between miR-1-ES cells and blunted fibrosis. However, MMP-9 expression was not significantly diminished in miR-1-ES cell

transplanted hearts relative to ES cell transplanted hearts. Our data indicates that although not significant, our miR-1-ES cells were able to parallel the anti-fibrotic effects of ES cells post-MI.

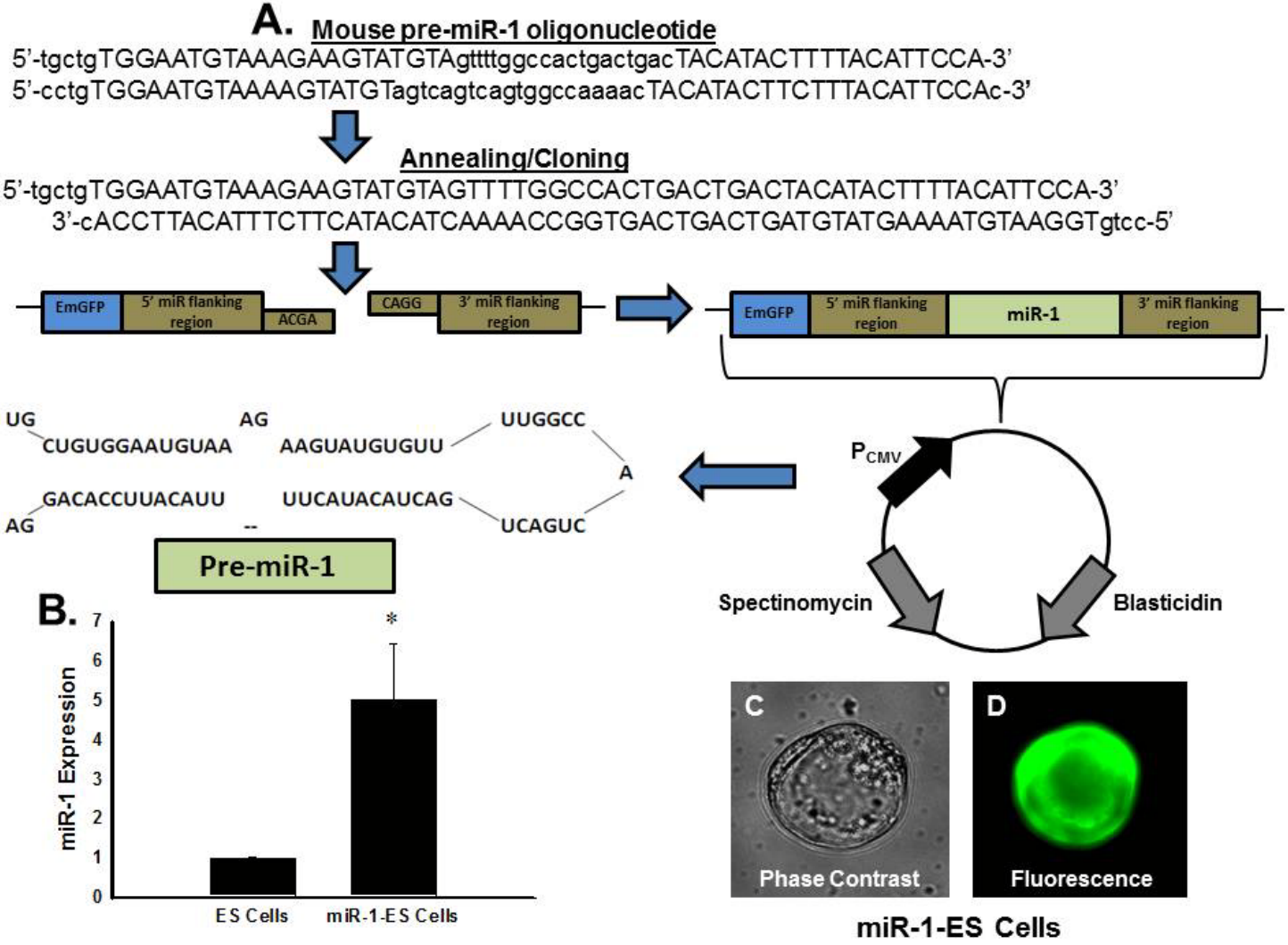
Finally, we needed to determine whether transplantation of miR-1-ES cells contribute to improved cardiac function following MI as the success of ES cell transplantation for post-MI therapy ultimately is defined by the improvement in cardiac contractility and output. In the present study, we have demonstrated that 2 weeks following MI, mice transplanted with miR-1-ES cells had significantly increased fractional shortening and ejection fraction compared to mice transplanted with ES cells. Conceivably, enhanced cardiac myocyte differentiation from transplanted miR-1-ES cells within the infarcted myocardium resulting in attenuated adverse remodeling contributed to improved overall cardiac function.

In conclusion, we report that; 1) miR-1 promotes ES cell differentiation into cardiac myocytes in the infarcted heart, 2) miR-1-ES cells inhibit cardiac myocyte apoptosis, 3) inhibited apoptosis mediated by miR-1-ES cells involves the PI3K/PTEN/Akt pathway, 4) inhibited apoptosis is also associated with attenuated oxidative stress in miR-1-ES cell transplanted hearts, 5) miR-1-ES cells inhibit interstitial and vascular fibrosis relative to control MI, and 6) transplanted miR-1-ES cells post-MI improve cardiac function.

Acknowledgements

The authors would like to thank Dr. Xilin Long for the generation of miR-1-ES cells, Reetu Singla for ES cell culture maintenance, Dr. Binbin Yan for assistance with confocal images, and Ajitha Dammalapati for histological assistance.

Figures


A.
Mouse pre-miR-1 oligonucleotide
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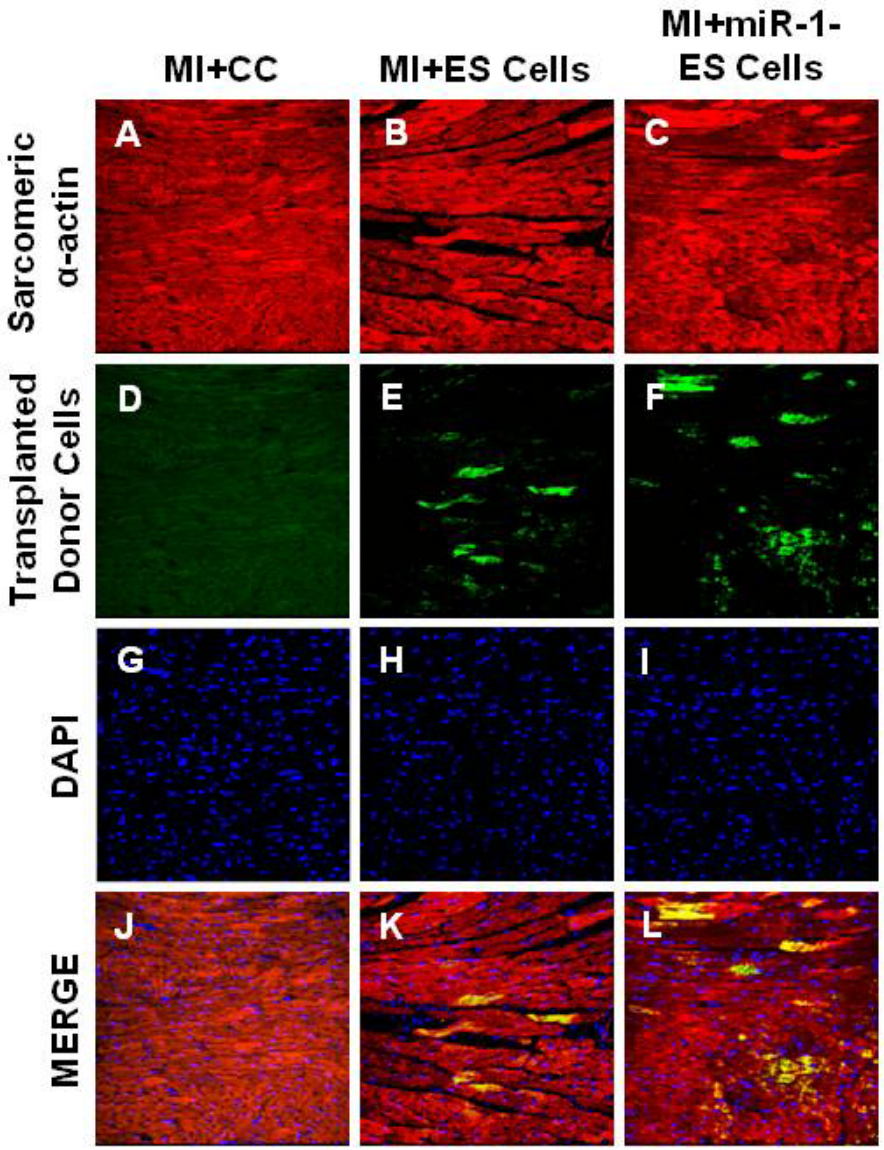
5'-cctgTGGAATGTAAAAGTATGTagtcagtcagtggccaaaacTACATACTTCTTTACATTCCAc-3'
Annealing/Cloning
5'-tgctgTGGAATGTAAAGAAGTATGTAGTTTTGGCCACTGACTGACTACATACTTTTACATTCCA-3'
3'-cACCTTACATTTCTTCATACATCAAAACCGGTGACTGACTGATGTATGAAAATGTAAGGTgtcc-5'
EmGFP
5' miR flanking region
ACGA
CAGG
3' miR flanking region
EmGFP
5' miR flanking region
miR-1
3' miR flanking region
UG
CUGUGGAAUGUAA
AG
AAGUAUGUGUU
UUGGCC
A
GACACCUUACAUU
AG
UUCAUACAUCAG
UCAGUC
Pre-miR-1
P CMV
Spectinomycin
Blasticidin
B.
miR-1 Expression
0
1
2
3
4
5
6
7
*
ES Cells
miR-1-ES Cells
C
Phase Contrast
D
Fluorescence
miR-1-ES Cells

Figure 1. miR-1-ES cell generation. A: Diagram showing mouse pre-miR-1 oligonucleotide cloned into an expression vector. GFP and miR-1 expression is driven by cytomegalovirus promoter. B: Histogram shows quantitative RT-PCR data demonstrating overexpression of miR-1 in miR-1-ES cells. Representative photomicrographs of miR-1-ES cells in bright field microscopy (C) and using fluorescence microscopy (D) to demonstrate GFP expression.


MI+CC
MI+ES Cells
MI+miR-1-ES Cells
Sarcomeric α-actin
Transplanted Donor Cells
DAPI
MERGE
A
B
C
D
E
F
G
H
I
J
K
L

M.

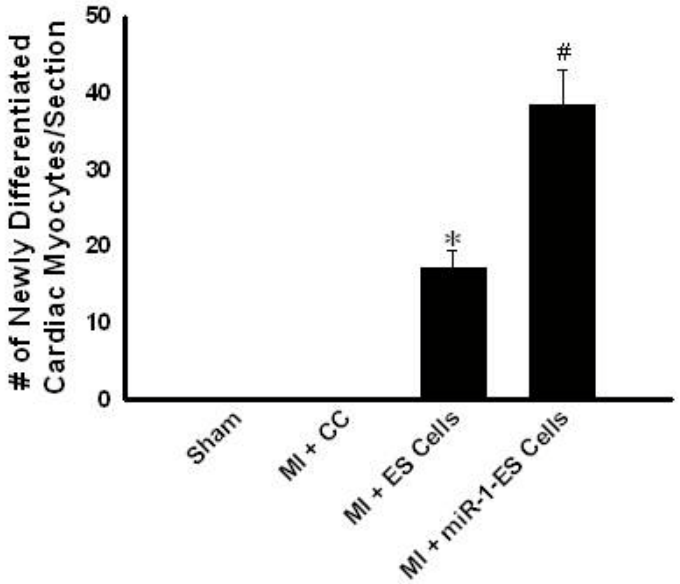
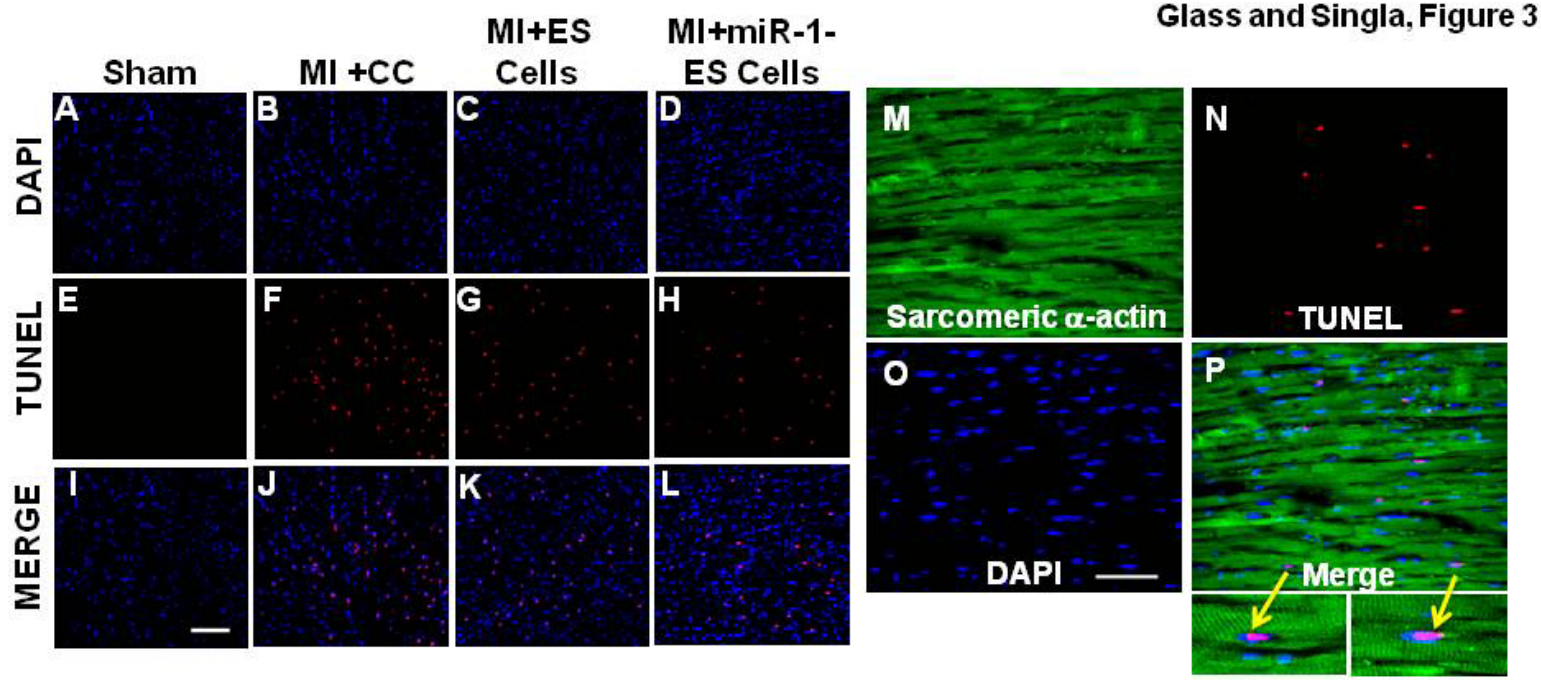
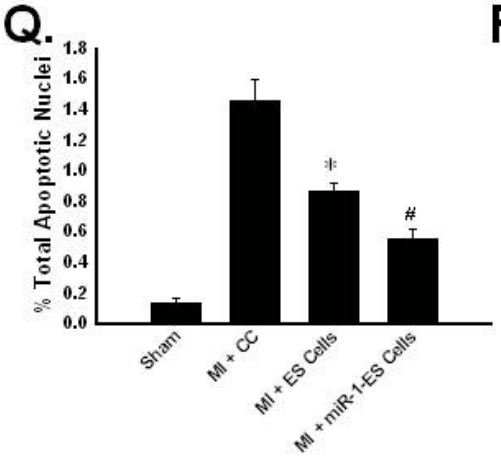
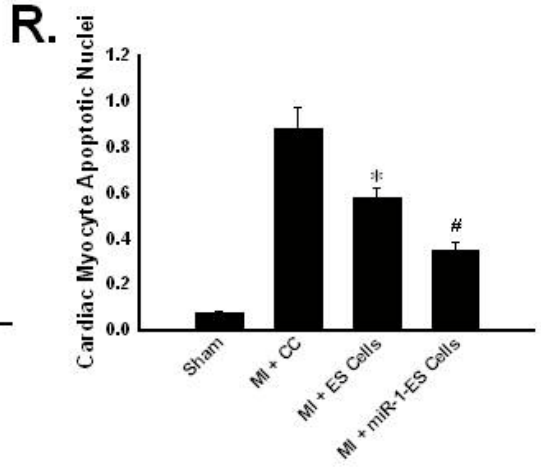

# of Newly Differentiated Cardiac Myocytes/Section
50
40
30
20
10
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 2. miR-1 enhances differentiation of ES cells into cardiac myocytes. Representative photomicrographs showing heart sections stained with anti-α-actin for cardiac myocytes (A-C), donor transplanted cells (D-F), nuclei stained with DAPI (G-I), and the merged image (J-L) showing co-expression of α-actin and GFP/RFP indicating differentiated donor cells. M: Histogram shows quantitative analysis of differentiated donor ES cells into cardiac myocytes 2 wks following MI. *p<0.01 vs. MI + CC, #p<0.001 vs. MI + ES cells and MI + CC. Data set are from n=8 different animals.

Glass and Singla, Figure 3







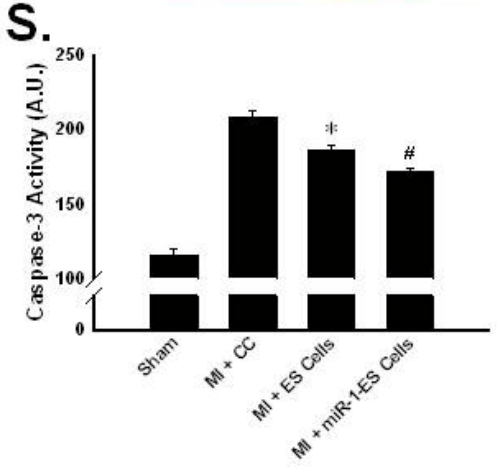


Figure 3. Effects of transplanted ES cells overexpressing miR-1 on host cardiac myocyte apoptosis in C57BL/6 mice. Representative photomicrographs of total nuclei stained with DAPI in blue (A-D), apoptotic nuclei stained with TUNEL in red (E-H), and merged nuclei in pink (I-L). Magnification, 40X. Scale bar = 50 μm. Top right panel (M-P), representative photomicrographs of section labeled with sarcomeric α-actin in green (M), TUNEL in red (N), DAPI in blue (O), and merged image (P) demonstrating apoptosis occurs within cardiac myocytes. Magnification, 40X. Boxed area below panel P enlarged to show co-localization of sarcomeric α-actin, TUNEL, and DAPI. Bottom left panel (Q), histogram shows quantitative total apoptotic nuclei. **p*<0.001 vs. MI + CC, #*p*<0.05 vs. MI + ES cells and MI + CC. Data set are from 1-2 sections from n=5-8 hearts/group. Bottom middle panel (R), histogram shows a significant decrease in cardiac myocyte apoptosis in miR-1-ES cell group. **p*<0.01 vs. MI + CC, #*p*<0.05 vs. MI + ES cells and MI + CC. Data set are from 1-2 sections from n=5-8 hearts/group. Bottom right panel (S), histogram shows quantitative decreases in caspase-3 activity in MI + miR-1-ES cells compared with MI + ES cells and MI + CC. **p*<0.01 vs. MI + CC, #*p*<0.05 vs. MI + ES cells and MI + CC. Data set are from heart homogenates of n=6-8 animals/group. A.U. = arbitrary units.

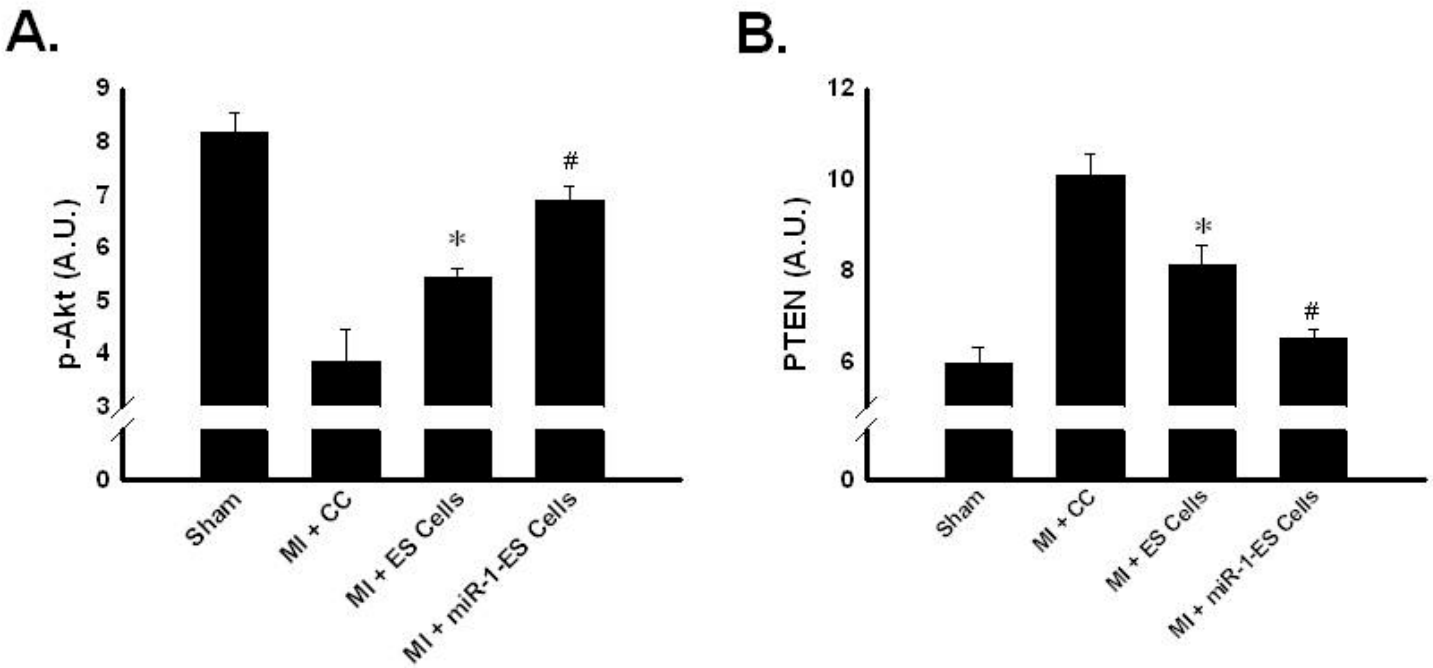
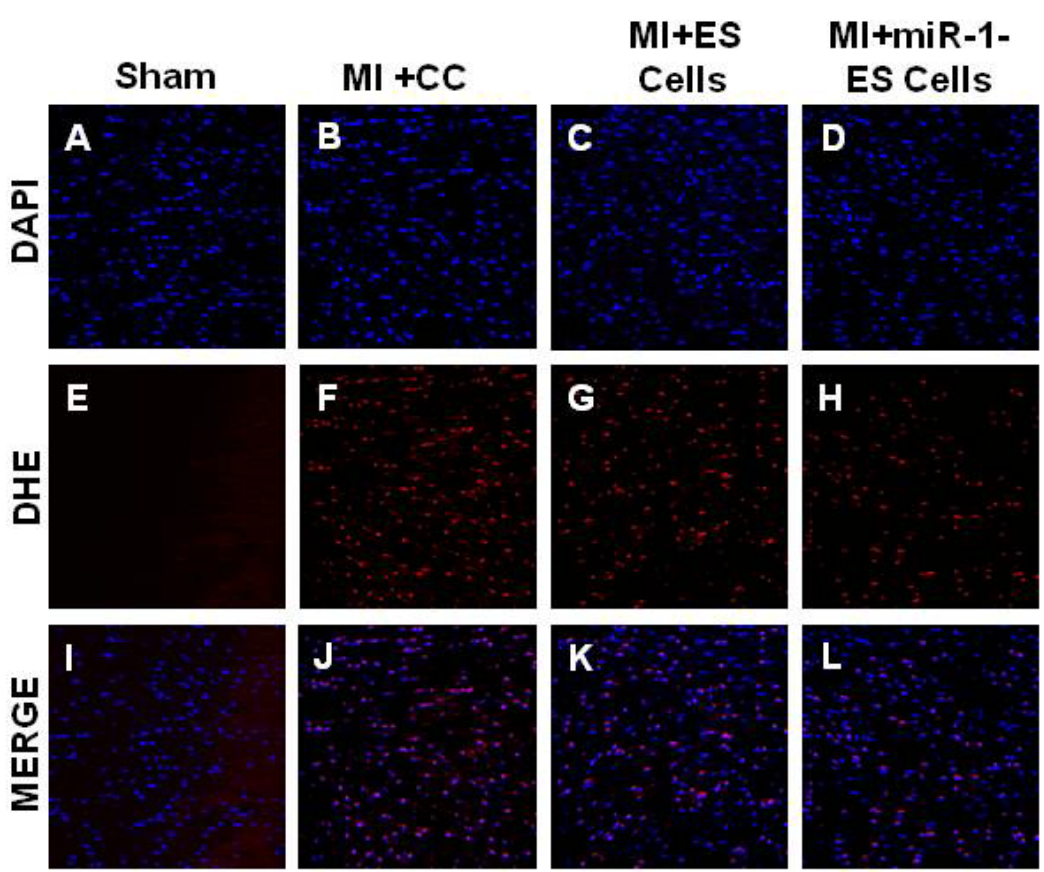

A.
p-Akt (A.U.)
9
8
7
6
5
4
3
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells
B.
PTEN (A.U.)
12
10
8
6
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 4. miR-1 mediates anti-apoptotic effects through modulation of the PTEN/Akt pathway. A: Histogram reveals significant increase in p-Akt activity in hearts transplanted with miR-1-ES cells. *p<0.05 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC. Data set are from heart homogenates of n=6-8 animals/group. B: Histogram show quantitative decrease in PTEN expression in hearts following transplantation of miR-1-ES cells. *p<0.05 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC. Data set are from heart homogenates of n=5-8 animals/group in duplicates. A.U. = arbitrary units.


Sham
MI +CC
MI+ES Cells
MI+miR-1-ES Cells
DAPI
DHE
MERGE
A
B
C
D
E
F
G
H
I
J
K
L

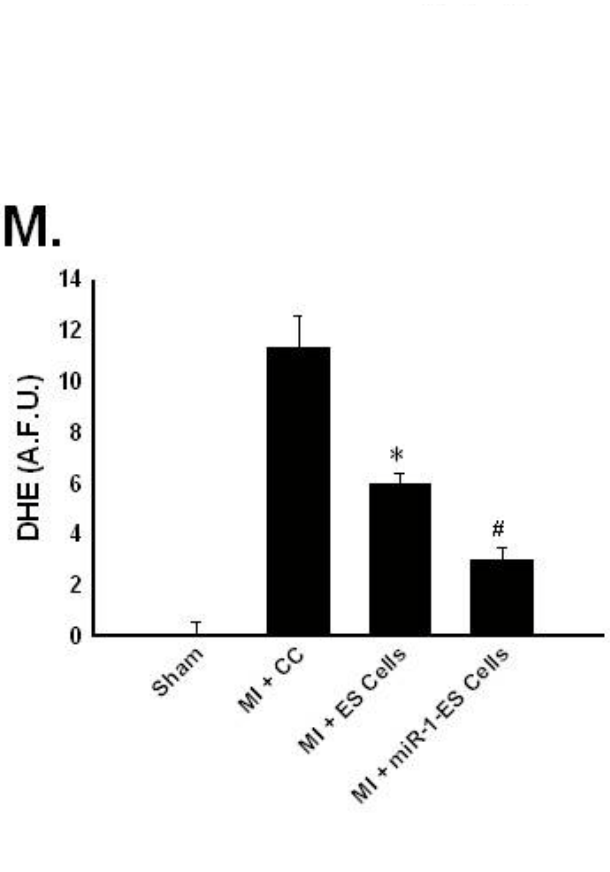

M.
DHE (A.F.U.)
14
12
10
8
6
4
2
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 5. miR-1-ES cells attenuate oxidative stress in the infarcted myocardium. Left panel (A-L), representative photomicrographs of DAPI in blue (A-D), DHE in red (E-H), and merged images (I-L) demonstrating a significant decrease in superoxide production in hearts transplanted with miR-1-ES cells. Magnification, 40X. Right panel (M), histogram shows quantitative analysis of DHE staining. *p<0.001 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC. Data set are from 1-2 sections from n=7-8 hearts/group. A.F.U. = arbitrary fluorescence units.

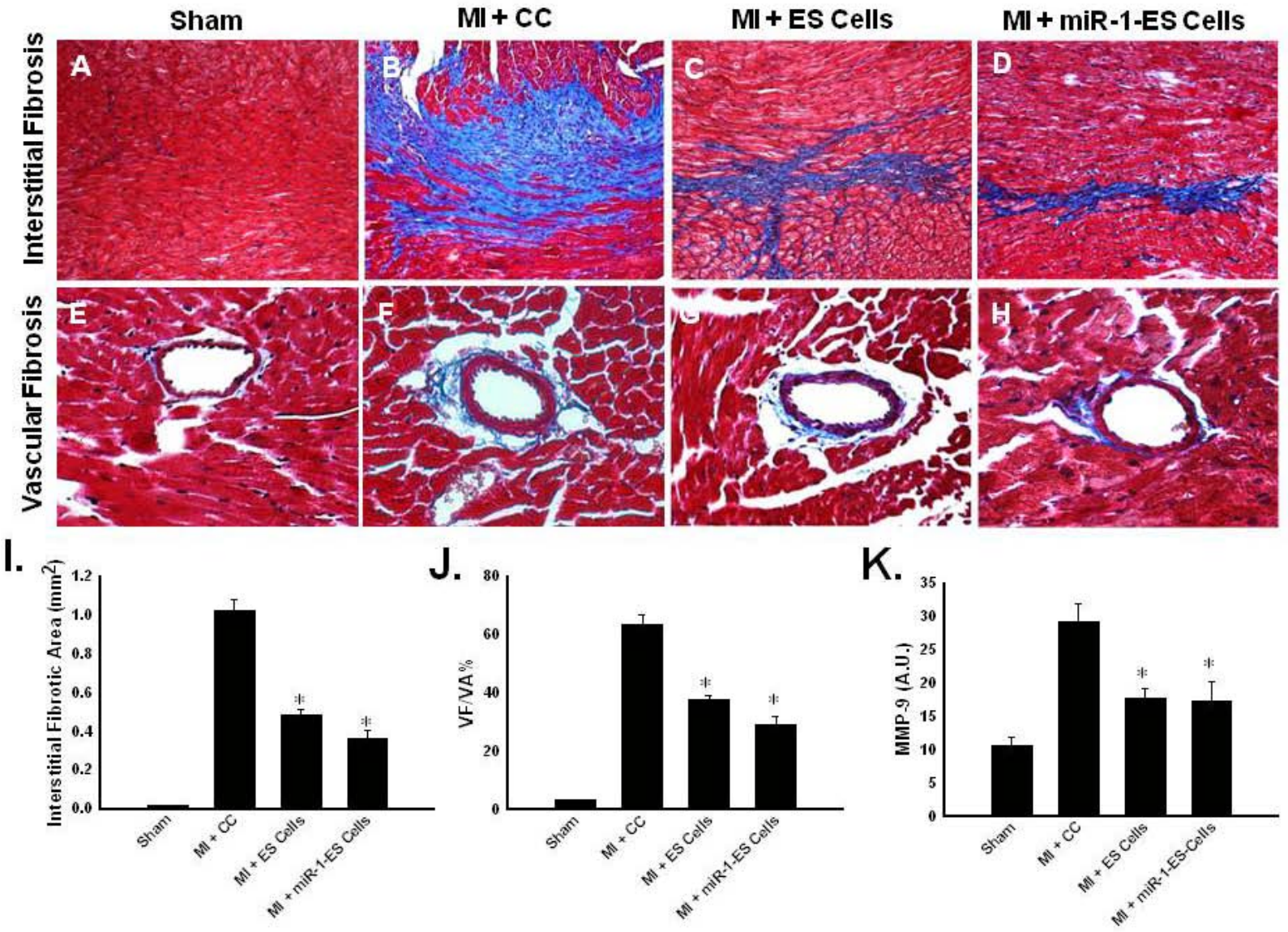

Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells
Interstitial Fibrosis
Vascular Fibrosis
A
B
C
D
E
F
G
H
I.
Interstitial Fibrotic Area (mm2)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells
J.
VF/VA %
80
60
40
20
0
K.
MMP-9 (A.U.)
35
30
25
20
15
10
5
0

Figure 6. Effects of transplanted ES cells overexpressing miR-1 on vascular and interstitial fibrosis. Representative photomicrographs from sections stained with Mason's trichrome 2 wks following coronary artery ligation from each group demonstrating interstitial (A-D) and vascular (E-H) fibrosis. Magnification, 40X. Bottom left panel (I), histogram shows quantitative interstitial fibrosis. *p<0.001 vs. MI + CC. Data set are from 1-2 sections from n=6-8 hearts/group. Bottom middle panel (J), histogram shows quantitative analysis of vascular fibrosis in heart sections after 2 wks of coronary artery ligation. *p<0.001 vs. MI + CC. VA= vessel area, VF=vessel fibrosis. Data set are 1-2 sections from n=6-8 hearts/group. Bottom right panel (K), histogram shows quantitative MMP-9 expression significantly decreased in miR-1-ES cell and ES cell groups. *p<0.01 vs. MI + CC. Data set are from heart homogenates of n=5-8 animals/group in duplicates.

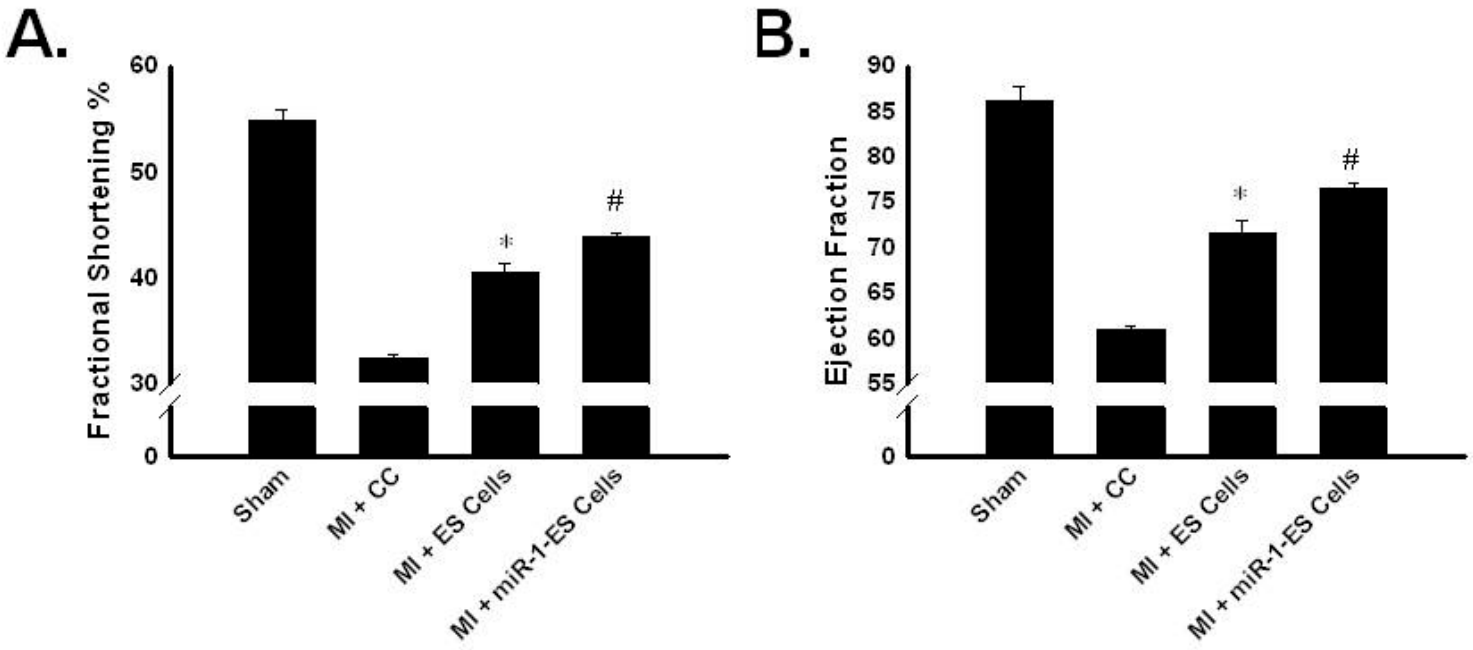

A.
Fractional Shortening %
60
50
40
30
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells
B.
Ejection Fraction
90
85
80
75
70
65
60
55
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 7. Transplanted miR-1-ES cells improve cardiac function in C57BL/6 mice following MI. Echocardiography was performed D14 following MI. A: Histogram shows average fractional shortening at 2 wks post-MI for all treatment groups. *p<0.001 vs. MI + CC, #p<0.01 vs. MI + ES cells and MI + CC. Data set are from n=8 animals/group. B: Histogram shows transplanted miR-1-ES cells significantly improve fractional shortening. *p<0.001 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC. Data set are from n=5-8 animals/group.

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CHAPTER THREE: ES CELLS OVEREXPRESSING microRNA-1 ATTENUATE APOPTOSIS IN THE INJURED MYOCARDIUM

Abstract

MicroRNAs (miRs) are small, single-stranded, non-coding RNA's involved in post-transcriptional negative gene regulation. Recent investigations have underscored the integral role of miRs in various biological processes including innate immunity, cell-cycle regulation, metabolism, differentiation, and cell death. In the present study, we overexpressed miR-1, a muscle-specific miR, in embryonic stem cells (miR-1-ES cells), transplanted them into the infarcted myocardium, and evaluated their impact on cardiac apoptosis and function. We provide evidence demonstrating reduced apoptosis following transplantation of miR-1-ES cells 4 weeks post-myocardial infarction as compared to respective controls assessed by TUNEL staining and a capsase-3 activity assay. Moreover, we show significant elevation in p-Akt levels and diminished PTEN levels in hearts transplanted with miR-1-ES cells as determined by enzyme-linked immunoassays. Finally, using echocardiography, we reveal mice receiving miR-1-ES cell transplantation post-myocardial infarction had significantly improved fractional shortening and ejection fraction compared with respective controls. Our data suggest transplanted miR-1-ES cells inhibit apoptosis, mediated through the PTEN/Akt pathway, leading to improved cardiac function in the infarcted myocardium.

Introduction

Myocardial infarction (MI) induces cardiac myocyte cell death triggering left ventricular remodeling leading to hypertrophy, fibrosis, and dysfunction(3; 21). Apoptosis, programmed cell death, is responsible for millions of cardiac myocytes lost

following MI and subsequent ventricular remodeling (3; 7). Taking into account cardiac myocytes are mostly terminally differentiated with minimal potential for cell division and the limited number of cardiac progenitor stem cells, intrinsic cardiac muscle regeneration is not sufficient to restore the heart to pre-MI homeostasis(15; 27). Given this, the challenge to develop effective therapies which protect the host myocardium from apoptosis following MI remains ever present.

Recent investigations have identified miRs as regulators of countless processes including cellular development, differentiation, metabolism, and death(4; 6; 9; 11). miRs are small, single stranded, non-coding RNAs which negatively influence gene expression through post-transcriptional modifications. miR manipulation to gain therapeutic effects for the treatment of various physiological and pathological conditions has gained notable attention within the research community.

Recently reported, miR-1 is a pro-cardiac miR which has been shown to enhance cardiac myocyte differentiation in the cell culture system(4; 28). Whether miR-1-overexpressing embryonic stem (ES) cells following transplantation into the infarcted myocardium can inhibit apoptosis 4 weeks post-MI requires investigation. Therefore, we postulate that miR-1 will act as an anti-apoptotic miR when overexpressed in transplanted ES cells post-MI. Our data suggests that miR-1 over expressing ES cells following transplantation into the infarcted myocardium reduce total apoptotic nuclei. Furthermore, the observed decrease in apoptosis is mediated through up regulation of the Akt pathway and down regulation of PTEN in miR-1-ES cell transplanted hearts. Finally, we illustrate that *in vivo* delivery of miR-1-ES cells 4 weeks post-MI improves cardiac function.

Materials and Methods

ES Cell Culture

Mouse CGR8 ES cells were passaged and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 15% ES-qualified FBS, leukemia inhibitory factor (LIF), glutamine, non-essential amino acids, penicillin/streptomycin, β-mercaptoethanol, and sodium pyruvate as previously described(23).

miR-1-ES Cell Generation

Pre-miR-1 oligonucleotides (5'-tgc tgT GGA ATG TAA AGA AGT ATG Tag ttt tgg cca ctg act gac TAC ATA CTT TTA CAT TCC A-3' and 5'-cct gTG GAA TGT AAA AGT ATG Tag tca gtc agt ggc caa aac TAC ATA CTT CTT TAC ATT CCA c-3') were cloned into an expression vector (pcDNA 6.2-GW/EmGFP, Invitrogen). 48 hrs post-transfection into mouse CGR8 ES cells using Lipofectamine 2000 (Invitrogen), media was changed and selection media containing 2 μg/ml blasticidin (Invitrogen) was added and changed every 48 hrs thereafter for 2-3 weeks. Blasticidin resistant ES cell colonies (labeled miR-1-ES cells) were passaged and maintained in ES cell culture medium as described beforehand. The miR-1-ES cell cloning strategy is part of another submitted paper (Glass and Singla, unpublished data).

RNA Extraction and real-time RT-PCR

Total RNA was extracted using RNA STAT-60 (Tel-Test). cDNA synthesis was performed with Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Specific primers (Assay ID# 002222, Applied

Biosystems) and Taqman® Universal PCR Master Mix (Applied Biosystems) were used for amplification and identification of miR-1. Real-time RT-PCR was performed using a CFX96™ Real-Time System (Bio Rad). The relative amount of miR-1 was normalized against U6 snRNA (Assay ID# 001973, Applied Biosystems) and the fold change for miR-1 was calculated.

MI and ES Cell Transplantation

All experimental procedures were performed on 8-10 week old C57BL/6 mice (Jackson laboratories) and were approved by the University of Central Florida animal review board. Male and female mice were divided into 4 study groups (n=8): Sham, MI + cell culture media (MI + CC), MI + ES cells, and MI + miR-1-ES cells. In brief, mice were intubated, ventilated using a rodent MiniVent (Harvard Apparatus), and anesthetized with 2.5% inhalant isoflurane throughout the procedure. Following a left thoracotomy, a permanent ligature was placed around the coronary artery. The peri-infarct region was identified and two separate intramyocardial injections totaling 20μl of media with or without 5.0×10^4 cells were delivered using a 29-gauge floating needle. After suturing the ribs, muscle, and skin, the lungs were expanded and mice were extubated. Sham animals received identical surgical procedures as detailed above while omitting the ligation. At D28 following surgery, mice were sacrificed using pentobarbital (80 mg/kg, ip) followed by cervical dislocation. Hearts were removed, transversely cut, and fixed in 4% paraformaldehyde for further analyses.

TUNEL Staining

Heart tissue fixed in 4% buffered formalin was embedded in paraffin, cut into serial sections (5 μm), and placed onto Colorfrost Plus slides (Fisher Scientific). TUNEL staining and analysis was performed as we previously reported(24; 25). In brief, deparaffinized heart sections were permeabilized with proteinase K (25 μg/ml in 100 mM Tris·HCl) and an in situ apoptotic cell death detection kit (TMR red; Roche Applied Biosystems) was used to identify apoptotic nuclei within the infarcted myocardium as per the manufacturer's instructions. Thereafter, heart sections were mounted with Anti-fade Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Sections were observed under Olympus fluorescent and confocal microscopes. The percentage of apoptotic nuclei was determined in the infarct and peri-infarct regions of heart tissue from n=5-8 animals/group. Percentage total apoptotic nuclei = (total number apoptotic nuclei) / (total number of DAPI) x 100%.

Caspase-3 Activity Assay

Caspase-3 activity was quantitated using a caspase-3 colorimetric activity assay kit from BioVision (K106-200). Heart tissue was homogenized in RIPA buffer containing phenylmethylsulfonyl fluoride, sodium orthovandate, protease inhibitor cocktail, and sodium fluoride. Following centrifugation, supernatant was removed and protein concentration was estimated for each sample using the Bradford assay. Caspase-3 activity assay was performed following the manufacturer's instructions included with the kit. The resulting ODs at 405 nm were normalized to the protein concentration of each

sample and plotted as arbitrary units (A.U.). Data was collected from heart homogenates of n=5-8 animals/group in duplicates.

p-Akt Activity Assay

p-Akt was quantified in heart homogenates using a phospho-Akt1 (PAN) ELISA kit (X1844k, Exalpha Biological) as detailed in the provided instructions. The developed color reaction was measured at 450 nm and the values obtained from the ELISA were normalized to the total protein concentration for each sample. Data was collected from heart homogenates of n=5-8 animals/group in duplicates.

PTEN ELISA

Phospho-PTEN (p-PTEN) was quantitated using a Phospho-PTEN Sandwich ELISA kit (#7285) from Cell Signaling Technology® as detailed in the provided manual. The developed color reaction was measured at 450 nm and results were corrected for the protein concentration of each tissue sample. p-PTEN data was obtained from heart homogenates of n=5-8 animals/group in duplicates and plotted as A.U.

Echocardiography

Cardiac function was assessed 4 weeks post-MI by non-invasive transthoracic echocardiography in short axis view at the mid-papillary muscle level using a Sonos 5500 Ultrasound system as we reported previously(25). Obtained measurements include left ventricular internal dimension-diastole (LVIDd), left ventricular internal dimension-systole (LVIDs), fractional shortening (FS), and ejection fraction (EF).

Statistical Analysis

Data are presented as a mean ± SEM. Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) and the Bonferroni test. $p<0.05$ was considered statistically significant.

Results

Following transfection of ES cells with our generated miR-1 expression vector, blasticidin resistant cells were selected and maintained in cell culture for 2-3 weeks. To determine miR-1 expression in miR-1-ES cells and parental ES cells, RT-PCR was performed. Our data demonstrates more than a seven-fold increase in mature miR-1 expression in miR-1-ES cells compared to parental ES cells (Figure 8).

Transplanted miR-1-ES Cells Inhibit Apoptosis Post-MI

To assess apoptosis within the infarcted myocardium, TUNEL staining was performed and representative photomicrographs are depicted in Figure 9A-L. At day 28 (D28), there was a significant decrease in TUNEL-positive nuclei post-MI in miR-1 ES cell transplanted hearts compared to ES cell and medium transplanted hearts ($p<0.05$, Figure 9M). To support our TUNEL data, caspase-3 activity was quantified and a significant decrease in hearts transplanted with miR-1-ES cells post-MI compared to hearts transplanted with ES cells or medium alone resulted ($p<0.05$, Figure 10).

miR-1 Enhances p-Akt Activation and Inhibits PTEN Post-MI

Several mechanisms of apoptosis inhibition through various cell survival cascades have been reported including activation of the Akt pathway(26). To

quantitatively assess phospho-Akt (p-Akt), we performed an ELISA and were able to circumvent hearts transplanted with miR-1-ES cells had significant p-Akt activation compared to hearts transplanted with ES cells and cell culture medium ($p<0.05$, Figure 11A). To evaluate whether enhanced p-Akt was consequent to inhibition of negative regulators of Akt activation, levels of PTEN, an antagonist of the Akt pathway, were quantitated. Our data demonstrates a significant reduction in PTEN in the miR-1-ES cell group vs. the ES cell or cell culture medium group ($p<0.01$, Figure 11B).

Transplanted miR-1-ES Cells Improve Cardiac Function Post-MI

Our echocardiography data at D28 illustrates mice receiving miR-1-ES cell transplantation following MI had significantly improved fractional shortening compared to ES cell and cell culture controls (mean ± SEM; MI + miR-1-ES cells: 44.73 ± 0.72, vs. MI + ES cells: 39.51 ± 1.02 and MI + CC 31.15 ± 1.69, $p<0.05$, Figure 12A). Additionally, miR-1-ES cell transplanted mice post-MI also had significantly improved ejection fraction when compared with controls post-MI (mean ± SEM; MI + miR-1-ES cells: 76.79 ± 0.86, vs. MI + ES cells: 70.46 ± 1.37 and MI + CC 61.36 ± 2.39, $p<0.05$, Figure 12B).

Discussion

Apoptosis, characterized by cell blebbing and shrinkage, chromatin condensation, and DNA fragmentation, plays an integral role in the pathogenesis of various cardiovascular diseases including MI(8). Cardiac myocyte apoptosis following MI is a highly orchestrated programmed cell death initiated through various stressors including cytokines, oxidative stress, and DNA damage(1; 7; 8). Although a multitude of

attempts have been made to attenuate apoptosis in the infarcted myocardium through the use of various stem cell populations and genetic modifications, apoptosis remains a key factor in the adverse outcomes of MI(5; 10; 12; 14; 22).

In this study, we reveal a novel role of miR-1, when over expressed in ES cells, in the regulation of cardiac myocyte apoptosis in the infarcted myocardium. We illustrate miR-1-ES cells inhibit host myocardium apoptosis 4 weeks post-MI. Conversely, recent investigations have reported that in the infarcted myocardium, miR-1 promotes apoptosis in cardiac myocytes(20; 29). However, the difference between the conflicting results lies within the cell type in which miR-1 is being expressed; that is, miR-1 has been shown to be apoptotic in endogenous cardiac cell types whereas we are suggesting miR-1 is anti-apoptotic when miR-1 over expressing ES cells are transplanted into the infarcted heart(29). Noticeably, these studies are unique from one another and demonstrate varying results. Nevertheless, this is not the first conflicting report in regards to the apoptotic nature of a particular miR. Consistent with our findings, it has been recently suggested that miRs can possess apoptotic, anti-apoptotic, or neither characteristic based solely on the type of cell in which they are being expressed(30). These conclusions underscore the importance of defining the apoptotic function of a distinct miR to a specific cell type.

We next wanted to define mechanisms by which transplanted miR-1-ES cells obviate host myocardium apoptosis post-MI. Previous studies have identified the involvement of the Akt pathway in the regulation of cell growth, metabolism, proliferation, and survival(17; 26). Moreover, the Akt pathway has been implicated in cardioprotection and apoptosis inhibition following MI through various secreted

paracrine factors including secreted fizzled related protein 2 and follistatin-like 1(2; 13; 16). We evaluated the levels of activated Akt and noted a negative correlation between Akt activation and apoptosis inhibition. Specifically, we were able to illustrate a significant increase in p-Akt paralleled by a significant decrease in apoptosis in miR-1-ES cell transplanted hearts compared to controls suggesting our data is in accordance with these previously published studies(2; 26). We propose that increased Akt expression observed within the miR-1-ES cell transplanted hearts leads to the observed cardioprotective effects through increased secreted paracrine factors. However, identification of exact paracrine mediators responsible for inhibited apoptosis is well beyond the scope of the current study and will require further investigation.

We next explored the potential effects of transplanted miR-1-ES cells on PTEN, an Akt signaling cascade inhibitor. We hypothesized that down regulation of PTEN might account for the up regulation of p-Akt observed in the miR-1-ES cell transplanted hearts. We were able to elucidate that levels of PTEN were, in fact, significantly decreased in hearts transplanted with miR-1-ES cells compared to control hearts. Although PTEN is not a predicted mRNA target of miR-1, further investigation may determine whether miR-1 down regulates PTEN activators thus explaining the increased Akt activity and decreased PTEN observed within this study. In fact, a recent publication demonstrated a mechanistic correlation between miR-101 and activation of Akt through down regulation of MAGI-2, a PTEN activator, as we suggest within the present study for miR-1(19).

Lastly, we tested how inhibition of apoptosis by transplanted miR-1-ES cells would impact cardiac function 4 weeks following MI. *In vivo* delivery of miR-1-ES cells

significantly increased fractional shortening and ejection fraction post-MI compared to respective controls. Conceivably, we suggest that inhibited apoptosis within the infarcted myocardium through miR-1 overexpression in transplanted ES cells obviated subsequent remodeling events post-MI leading to improved cardiac function. Our data is in agreement with a previously published study suggesting *in vivo* delivery of miR-24 into the mouse infarcted heart suppressed apoptosis thus attenuating contractility loss(18).

Next our data suggest that the observed beneficial effects in the present study may not be the direct effects of released miR-1 from overexpressing ES cells on reduced apoptosis. Rather, it may involve various factors released from transplanted miR-1-ES cells. Therefore, transplantation of LNA-miR-1 directly into the infarcted heart in comparison with the overexpressing miR-1-ES cells may further shed light on the mechanisms of reduced apoptosis in this model.

In summary, we report that transplanted miR-1-ES cells inhibit apoptosis in the infarcted heart and these effects are mediated through the PTEN/Akt pathway. Furthermore, we illustrate the capability of transplanted miR-1-ES cells to improve cardiac function 4 weeks post-MI. Our data suggests the potential therapeutic applications using miR-1 modulation for the treatment of apoptosis-induced cardiac conditions. However, to determine how miR-1 exerts its anti-apoptotic effects in infarcted myocardium when overexpressed in ES cells, further studies are required to determine which mRNA targets are directly regulated by miR-1.

Acknowledgements

This work was supported in part from grants from the National Institutes of Health [1R01HL090646-01, and 5R01HL094467-02 to DKS].

Figures

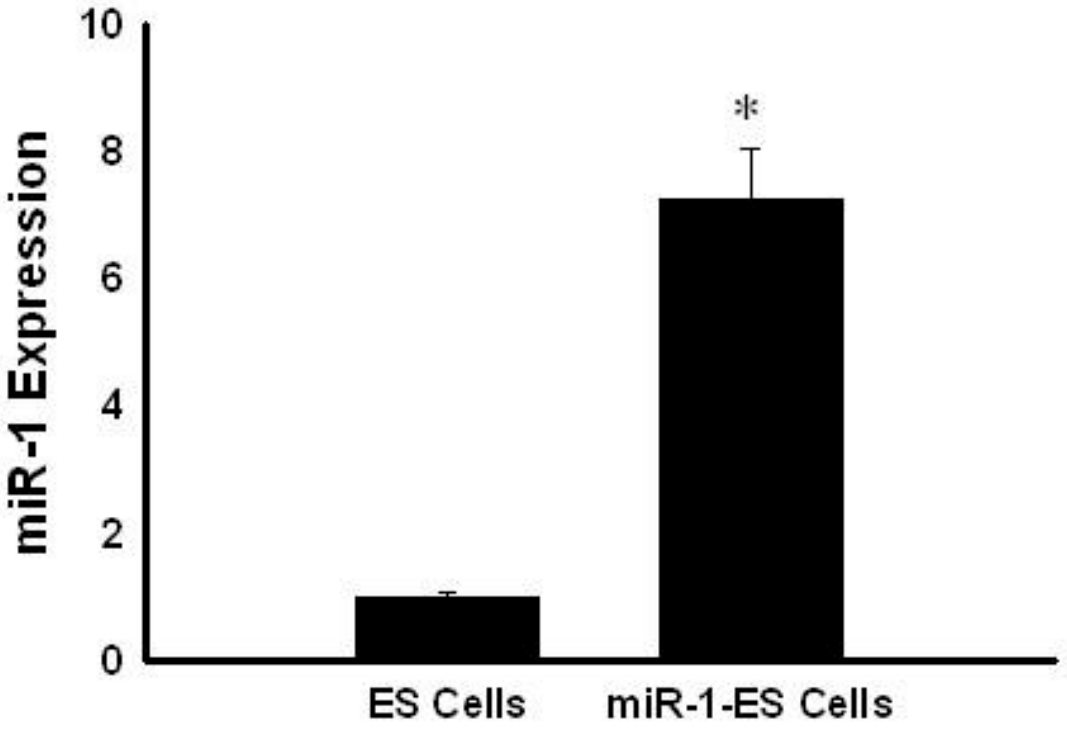
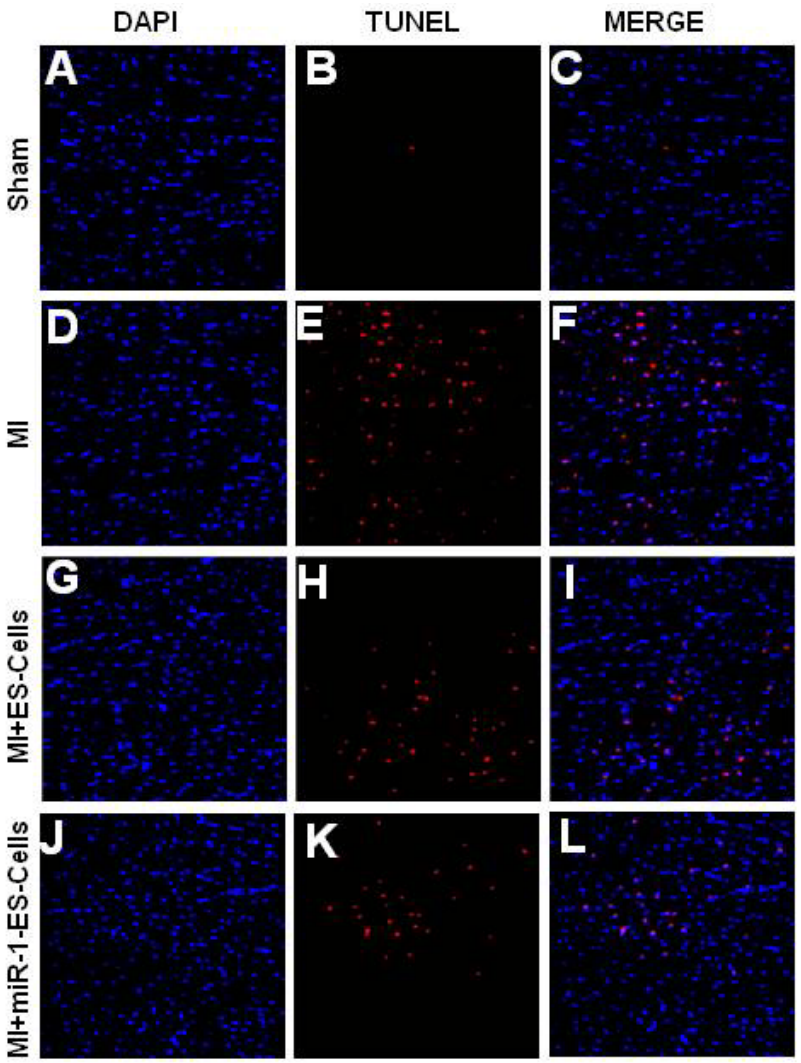


Figure 8. miR-1 overexpression confirmed by RT-PCR. Histogram shows quantitative RT-PCR data demonstrating overexpression of miR-1 in miR-1-ES cells. *p<0.001 vs. ES cells.


DAPI
TUNEL
MERGE
Sham
MI
MI+ES-Cells
MI+miR-1-ES-Cells
A
B
C
D
E
F
G
H
I
J
K
L

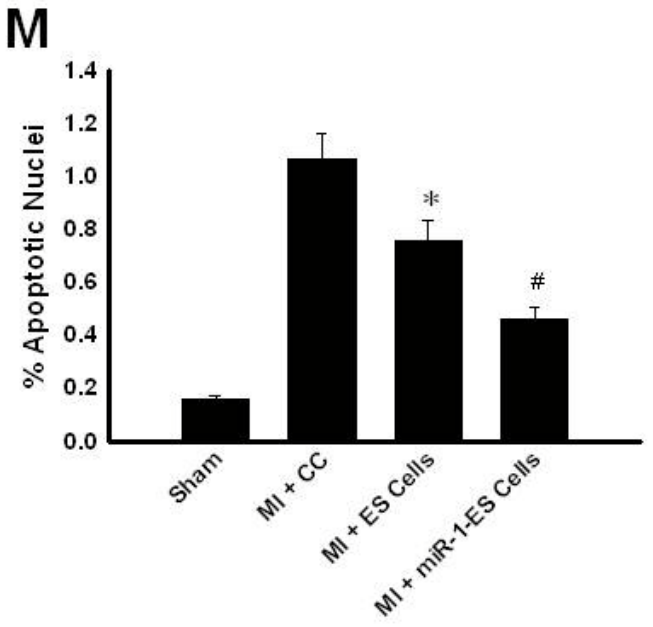

M
% Apoptotic Nuclei
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 9. Transplanted miR-1-ES cells inhibit apoptosis. Representative photomicrographs of DAPI in blue (A-J), apoptotic nuclei in red (B-K), and merged nuclei in pink (C-L). Magnification, 40X. Right histogram (M) shows quantitative apoptotic nuclei. *p<0.05 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC.

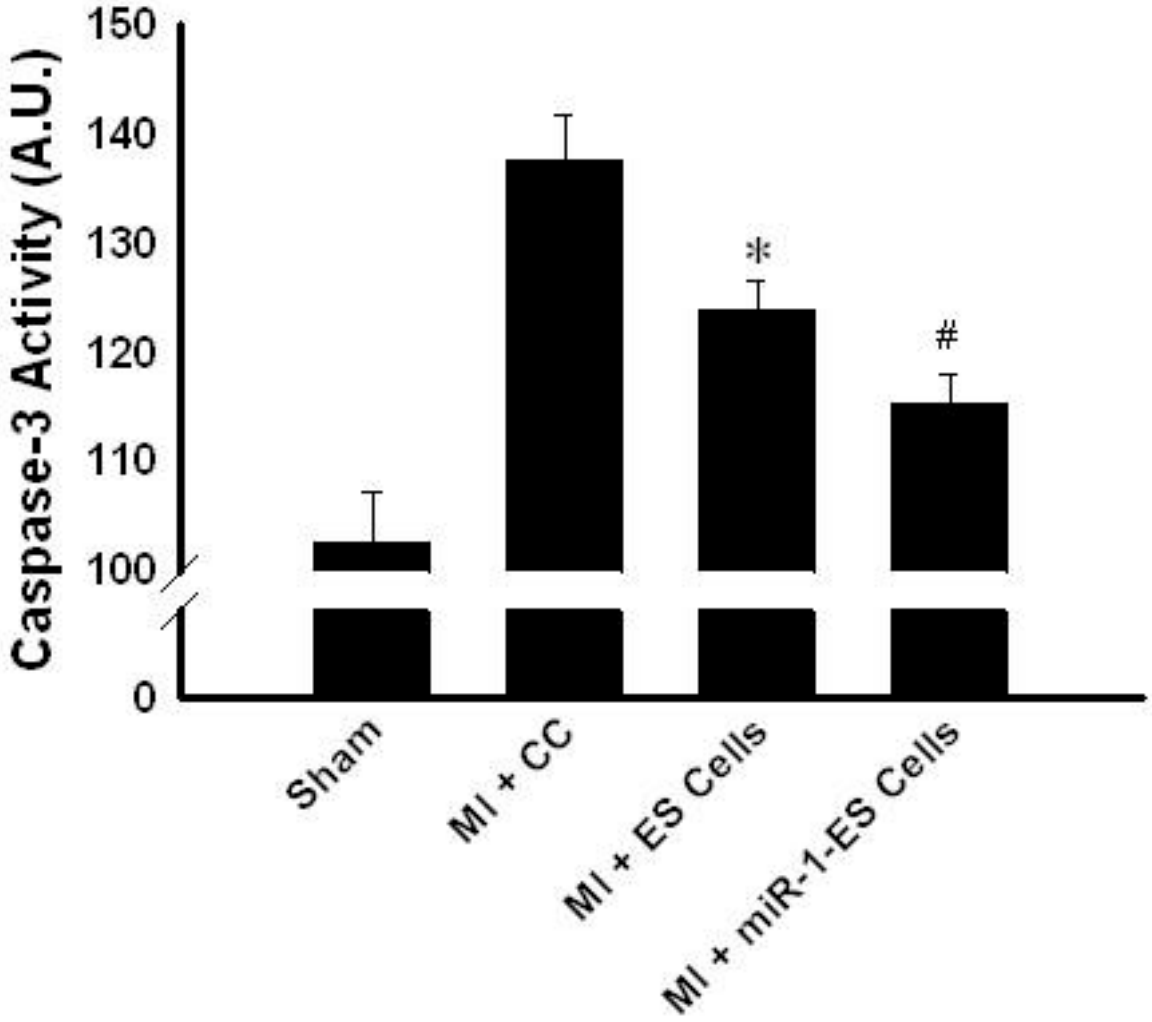

Caspase-3 Activity (A.U.)
150
140
130
120
110
100
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 10. miR-1-ES cells inhibit caspase-3 activity in the infarcted myocardium. Histogram shows significant decreases in caspase-3 activity in MI + miR-1-ES cells compared with MI + ES cells and MI + CC. *p<0.001 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC.

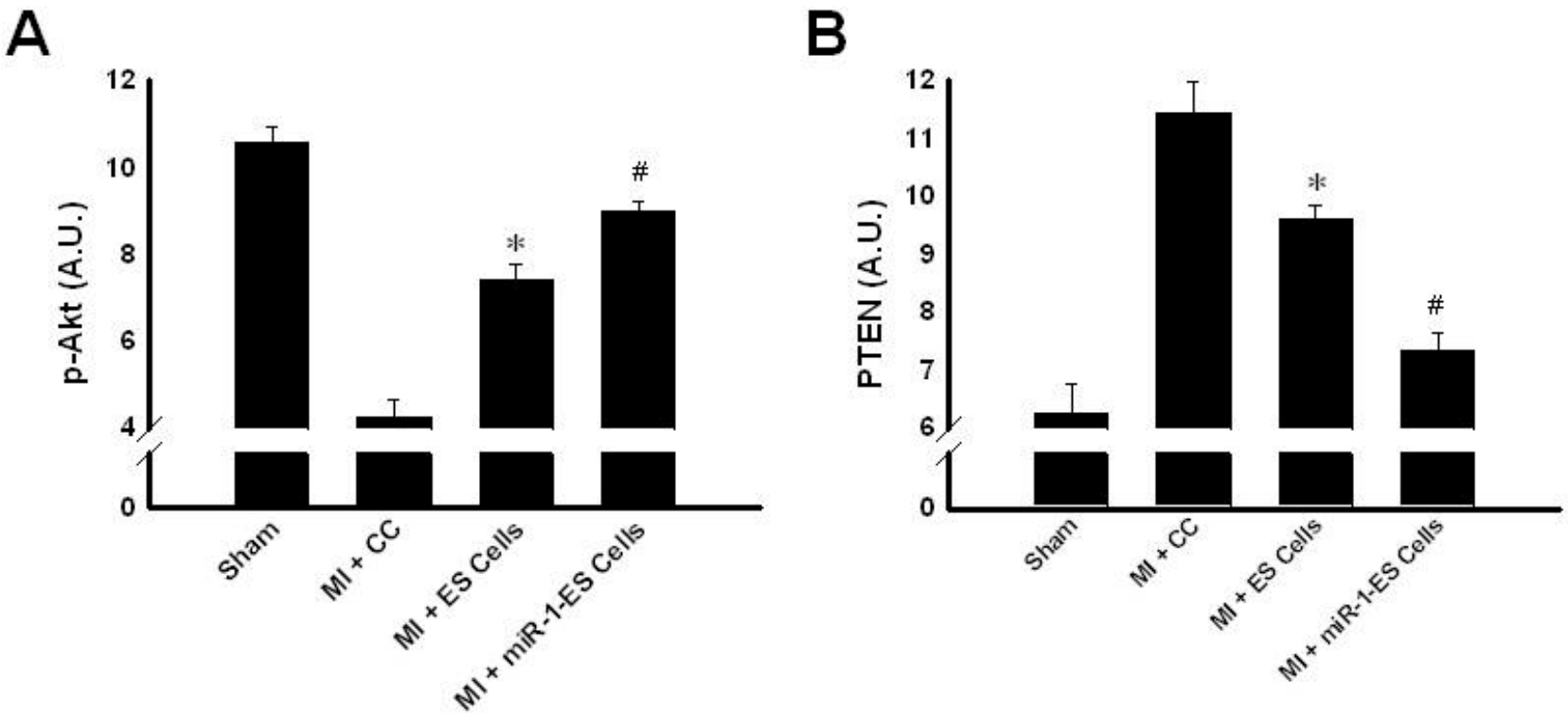

A
p-Akt (A.U.)
12
10
8
6
4
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells
B
PTEN (A.U.)
12
11
10
9
8
7
6
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 11. miR-1-ES cells exert apoptosis inhibition through PTEN/Akt pathway. Left histogram (A) reveals significant increase in p-Akt activity in hearts transplanted with miR-1-ES cells. *p<0.001 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC. Right histogram (B) show quantitative PTEN expression. *p<0.05 vs. MI + CC, #p<0.01 vs. MI + ES cells and MI + CC.

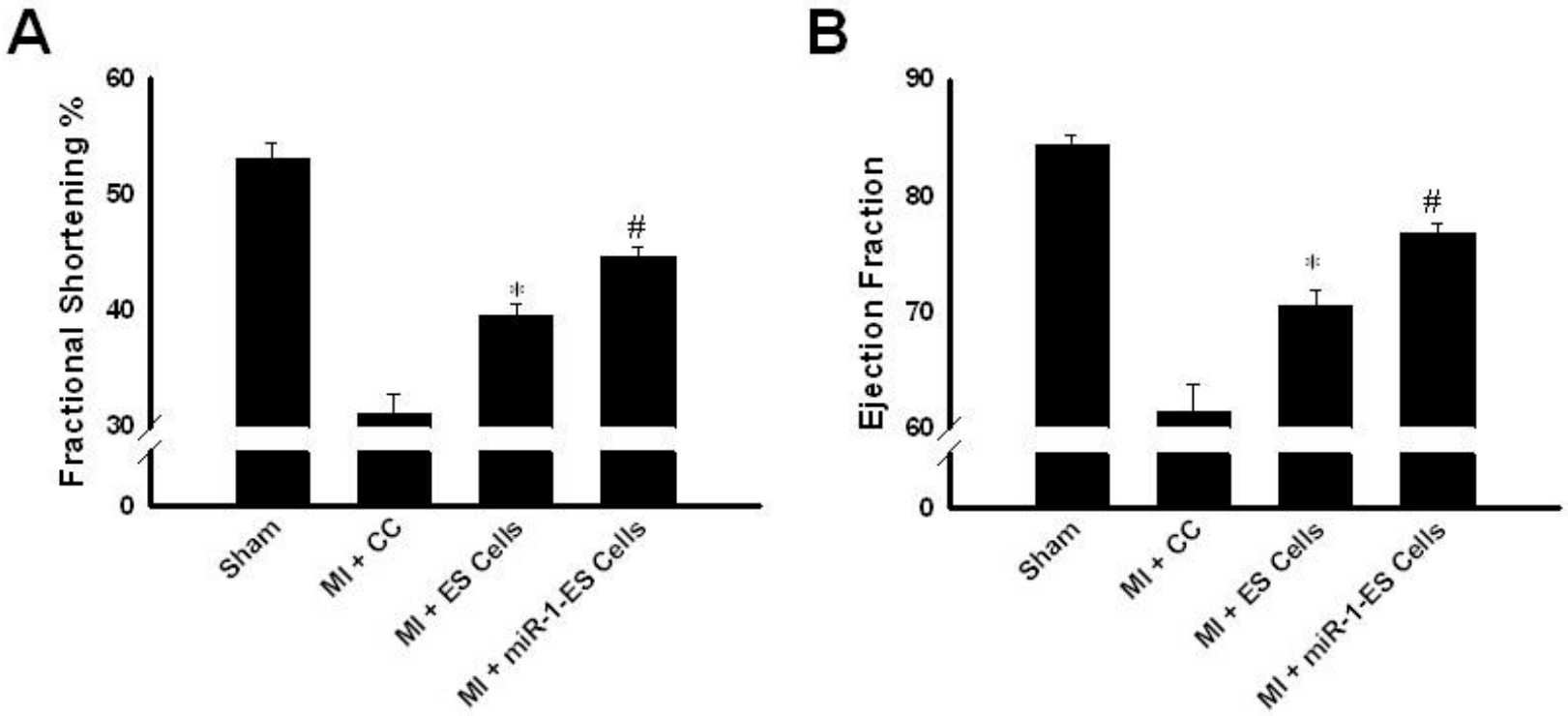

A
Fractional Shortening %
60
50
40
30
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells
B
Ejection Fraction
90
80
70
60
0
*
#
Sham
MI + CC
MI + ES Cells
MI + miR-1-ES Cells

Figure 12. Transplanted miR-1-ES cells improve cardiac function four weeks following MI. Echocardiography was performed at four weeks following MI. Left histogram (A) shows average fractional shortening for all treatment groups. *p<0.001 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC. Right histogram (B) shows transplanted miR-1-ES cells significantly improve fractional shortening. *p<0.01 vs. MI + CC, #p<0.05 vs. MI + ES cells and MI + CC.

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CHAPTER FOUR: OVEREXPRESSION OF TIMP-1 IN EMBRYONIC STEM CELLS ATTENUATES ADVERSE CARDIAC REMODELING FOLLOWING MYOCARDIAL INFARCTION

Abstract

Transplanted embryonic stem (ES) cells following myocardial infarction (MI) contribute to limited cardiac repair and regeneration with improved function. Therefore, novel strategies are still needed to understand the effects of genetically modified transplanted stem cells on cardiac remodeling. The present study evaluates whether transplanted ES cells overexpressing TIMP-1, an anti-apoptotic and anti-fibrotic protein, can inhibit native cardiac myocyte apoptosis, reduce fibrosis, and enhance cardiac function in the infarcted myocardium. MI was produced in C57BL/6 mice by coronary artery ligation. TIMP-1-ES cells, ES cells, or culture medium (control) was transplanted into the peri-infarct region of the heart. Immunofluorescence, TUNEL staining, caspase-3 activity, histology, and echocardiography were used to assess apoptosis, fibrosis, and heart function. Hearts transplanted with TIMP-1-ES cells demonstrated a reduction in apoptosis as well as an increase ($p<0.05$) in p-Akt activity compared with ES cells or culture media controls. Interstitial and vascular fibrosis was significantly ($p<0.05$) decreased in the TIMP-1-ES cell group compared to controls. Furthermore, MMP-9, a key pro-fibrotic protein, was significantly ($p<0.01$) reduced following TIMP-1-ES cells transplantation. Echocardiography data showed fractional shortening and ejection fraction were significantly ($p<0.05$) im*p*roved in the TIMP-1-ES cell group compared with respective controls. Our data suggest that transplanted ES cells overexpressing TIMP-1 attenuate adverse myocardial remodeling and improve cardiac function compared with ES cells which may have therapeutic potential in regenerative medicine.

Introduction

Cell transplantation strategies using renewable sources of cardiac myocytes to repair damaged myocardium, replace lost cardiac cells, and prevent remodeling following MI has gained significant attention. We, as well as others, have shown that transplanted embryonic stem (ES) cells post-MI have the potential to engraft, differentiate into cardiac-specific cell types, limit adverse myocardial remodeling, and improve heart function(3; 9; 22; 23). However, inhibition of adverse cardiac remodeling following cell transplantation is minimal relative to normal heart architecture and overall cardiac function. Therefore, novel approaches are needed to augment the efficacy by which transplanted ES cells enhance cardiac repair in order to optimize cardiac regain-of-function following cell transplantation therapy.

Following MI, ventricular architectural remodeling consequent to myocardial infarction (MI) is characterized by various physiological and cellular changes including cardiac cell death via apoptosis and necrosis, fibrosis, and hypertrophy leading to end stage heart failure(2; 10; 13; 18; 23). In response to myocardial cell death post-MI, increased expression of extracellular matrix (ECM) proteins, specifically collagen type I and III, occurs evoking fibrosis formation(16; 28). Fibrosis within the injured myocardium causes stiffening of the heart and overall systolic and diastolic dysfunction. Matrix metalloproteinases (MMPs) are a family of endopeptidases which play a fundamental role in the degradation and aberrant production of ECM proteins leading to alterations in ventricular architect post-MI. Tissue inhibitors of metalloproteinases (TIMPs) are innate protease inhibitors of MMPs. TIMP-1, the most extensively characterized TIMP, is not only a well-documented anti-fibrotic protein(4; 5; 7), but also has recently been shown to

exhibit anti-apoptotic characteristics in non-cardiac cell types such as breast epithelial cells (15; 24; 31). Moreover, we were the first to report that TIMP-1 is a cytoprotective released factor from ES cells which prevents H_2O_2-induced apoptosis *in vitro* in cardiomyoblasts (H9c2 cells)(24). However, whether TIMP-1, when overexpressed in transplanted ES cells, can impact adverse cardiac remodeling post-MI by blunting host myocardium apoptosis and fibrosis has yet to be delineated.

In the present study, we hypothesize that ES cells overexpressing TIMP-1 (TIMP-1-ES cells) following transplantation into the infarcted myocardium will attenuate apoptosis and fibrosis along with improve cardiac function. To accomplish this hypothesis, we generated ES cells overexpressing TIMP-1 (TIMP-1-ES cells), transplanted them into the infarcted myocardium, and evaluated their impact on apoptosis, fibrosis, and associated cardiac function. Our data shows that transplanted TIMP-1-ES cells post-MI significantly inhibit cardiac myocyte apoptosis compared with ES cells and the anti-apoptotic effect of TIMP-1 is, in part, mediated through the Akt pathway. Furthermore, we demonstrate that transplanted TIMP-1-ES cells inhibit both interstitial and vascular fibrosis in the infarcted myocardium. Finally, we show that transplantation of TIMP-1-ES cells post-MI significantly enhances cardiac function.

Materials and Methods

ES Cell Culture

CGR8 ES cells with or without TIMP-1 were cultured and maintained on 0.1 % gelatin-coated tissue culture plates in Dulbecco's minimum essential medium (DMEM) growth media supplemented with leukemia inhibitory factor (LIF), sodium pyruvate,

glutamine, penicillin/streptomycin, non-essential amino acids, β-mercaptoethanol, and 15% ES qualified fetal bovine serum as we previously reported(27).

TIMP-1-ES Cell Generation

The mammalian expression vector (pTurboFP635-C) encoding red fluorescent protein (RFP) was purchased from Evrogen (Moscow, Russia). The cDNAs coding mouse TIMP-1 gene were generated by PCR cloning using high fidelity DNA polymerase and PCR primers (forward: 5'-AGAGCAGATACCATGATG-3' and reverse: 5'-GAAGGCTTCAGGTCATCG-3'). The PCR product was cloned to the Bgl II and BamH l site of the pTurboFP635 C-terminus as a single open reading frame. The expression vector contained a neomycin-resistance gene for selection of stable transfectants in CGR8 ES cell lines. The sequence of mouse TIMP-1 gene was confirmed by restriction enzyme digestion and DNA sequencing analysis.

CGR8 mouse ES cells were transfected with the TIMP-1 expression vector using Lipofectamine 2000 (Invitrogen). Selection media containing 100 μg/ml G418 (Invitrogen) was added 48 hrs post-transfection. Selection media was changed every 48 hrs and cells were maintained for 2-3 weeks. Thereafter, G418 resistant ES cell colonies (labeled TIMP-1-ES cells) were selected, expanded, and maintained in ES cell culture medium. TIMP-1-ES cell clones were confirmed with elevated TIMP-1 protein expression as compared to parental CGR8 ES cells through western blot analysis using a primary antibody against TIMP-1 (sc-5538, Santa Cruz) following by incubation with goat anti-rabbit IgG secondary antibody (sc-2004, Santa Cruz).

MI and ES Cell Transplantation

All mice were maintained and used as approved by the institutional animal review board. C57BL/6 mice (8-10 weeks old) were divided into 4 study groups (n=8 per group): Sham, MI + cell culture media (MI + CC), MI + ES cells, and MI + TIMP-1-ES cells. In brief, mice were anesthetized, endotracheally intubated, and ventilated using a rodent MiniVent (Harvard Apparatus). Anesthesia was maintained using 2.5% isoflurane throughout the procedure. Left thoracotomy was performed and the descending left coronary artery was ligated as we reported previously(12). Following ligation, two intramyocardial injections of 10μl of medium containing $2.5X10^4$ TIMP-1-ES cells or ES cells was delivered into the peri-infarct region. The chest was then closed, lungs expanded, and mice were weaned from the ventilator and extubated. Sham group animals received all identical surgical procedures with the exception of no coronary artery ligation. Mice received postoperative care, including analgesia (buprenorphine, 0.5 mg/kg), for 2 days or until normal behavior resumed. Two weeks following surgery, mice were sacrificed by pentobarbital (80 mg/kg) followed by cervical dislocation. Hearts were removed, transversely cut, and fixed in 4% paraformaldehyde for further evaluation.

Histopathology

Heart tissue was embedded and sectioned as previously described(23). In brief, heart tissue was embedded in paraffin blocks, cut in serial sections (5 μm), and placed onto Colorfrost Plus microscope slides (Fisher Scientific). Heart sections were deparaffinized using room temperature xylene followed by sequential incubation in 100,

95, and 70% ethanol for 3 minutes and washed with distilled water. Heart sections were then stained with Mason's trichrome. To quantify interstitial fibrosis, infarct, peri-infarct, and non-infarct regions of the heart were examined and fibrosis was quantified by measuring the total blue area/total heart area x 100%. Vascular fibrosis was quantified by measuring vascular fibrosis/vessel area x 100% in 1-2 sections from 5-8 hearts/group. All fibrosis measurements were made using NIH image J software.

TUNEL and Cardiac α-Actin Staining

TUNEL staining was performed as previously described(25). In brief, heart sections were deparaffinized and permeabilized with proteinase K (25 μg/ml in 100 mM Tris·HCl). The TUNEL assay kit (TMR red; Roche Applied Biosystems) was used as per the manufacturer's instructions to detected host myocardium apoptotic nuclei. Apoptotic cells were co-labeled with cardiac α-actin (A2172, Sigma) to detected apoptosis within cardiac myocytes. Heart sections were then mounted with Antifade Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) for nuclear staining and observed under Olympus and confocal microscopes. Percentage total apoptotic nuclei = (total number of red stained apoptotic nuclei) / (total number of blue stained nuclei with DAPI) x 100. Additionally, the percentage of apoptotic nuclei in cardiac myocytes was also determined by (total number of red stained apoptotic nuclei co-localized with sarcomeric α-actin) / (total number of blue stained nuclei with DAPI) x 100. Total apoptotic nuclei and apoptotic cardiac myocyte nuclei were quantitated in the infarct and peri-infarct regions of the

heart in 1-2 sections from 5-8 hearts from each group. These cell counts were performed as previously reported by us and others(6; 8; 26).

Caspase-3 Activity Assay

Caspase-3 activity was quantitated using a caspase-3 colorimetric activity assay kit (K106-200, BioVision). Hearts were homogenized in RIPA buffer containing protease inhibitor cocktail (Sigma), phenylmethylsulfonyl fluoride (PMSF) (1mM), sodium orthovandate (2mM), and sodium fluoride (5mM), collected, and centrifuged at 11766 rpms for 5 min. The supernatant was removed and protein concentration estimation was performed using a Bio Rad assay. Manufacturer's instructions were followed to complete the activity assay. Developed blue color was measured in a microtiter plate reader (Bio Rad) at 405 nm. Caspase-3 activity graph was plotted as arbitrary units (A.U.).

Phosphorylated Akt Activity Assay

Phosphorylated Akt (p-Akt) was quantitated using a phospho-Akt1 (PAN) ELISA kit (X1844k, Exalpha Biological) as previously described and detailed in the instruction manual provided with the kit(26). In brief, hearts were homogenized and proteins were estimated by the Bradford method. Samples (25 μl) and sample diluents (75 μl) were added to the wells labeled with captured antibodies provided in the kit for 2 hrs. Following washing, samples were incubated with detector antibody for 2 hrs, HRP conjugate for 30 mins, and substrate for 30 mins with washings in between each step. A stop solution was added and developed yellow color was measured in a microtiter plate reader (Bio Rad) at 450 nm. Phospho-Akt graph was plotted as detailed in the kit.

MMP-9 Immunoassay

MMP-9 concentration was determined by an enzyme-linked immunoassay (MMPT90, R&D Systems). In brief, hearts from n=8 animals/group were homogenized and protein concentrations were estimated as aforementioned. Samples were loaded into monoclonal MMP-9 immobilized antibody-coated wells provided in the kit for 2 hrs. Following washings, a conjugate (polyclonal antibody for mouse MMP-9) was added for an additional 2 hrs. Again the wells were washed and a substrate solution was added to each well for 30 minutes followed by the addition of a stop solution. A color reaction developed proportional to the amount of bound MMP-9 and was measured in a microtiter plate reader (Bio Rad) at 450 nm. MMP-9 graph was plotted as arbitrary units (A.U.).

Echocardiography

Cardiac function for all mice groups was assessed non-invasively by transthoracic echocardiography at D14. Mice were anesthetized with 2% isoflurane, placed and maintained on a temperature controlled heating pad, and the chest was shaved. Echocardiography was performed using a Phillips Sonos 5500 Ultrasound system with a 15-6L hockey stick transducer. Left ventricular internal dimension-diastole (LVIDd), left ventricular internal dimension-systole (LVIDs), fractional shortening (LVIDd-LVIDs/LVIDd x 100), and ejection fraction ((left ventricular volume at end diastole (EDV) – left ventricular volume at end systole (ESV))/EDV, EDV and ESV were calculated using the Teichholz formula(29)) were determined at the mid-papillary muscle level in short axis view.

Statistical Analysis

All values presented as a mean ± SEM. Statistical analysis was performed using the students' t-test, one-way analysis of variance (ANOVA), and the Bonferroni test. Statistical significance was assigned when $p < 0.05$.

Results

To elucidate the impact of TIMP-1 in transplanted ES cells post-MI on cardiac remodeling and function, we overexpressed TIMP-1 (Figure 13A-C) in CGR8 ES cells. Following selection with G418, TIMP-1-ES cells were maintained in cell culture for 10-12 weeks. To determine stable transfection, western blot analysis was performed and our data shows significantly increased TIMP-1 expression compared to parental ES cells ($p<0.001$, Figure 13D).

Transplanted TIMP-1-ES Cells Prevent Apoptosis in the Infarcted Heart

To determine whether transplanted ES cells overexpressing TIMP-1 inhibit apoptosis compared to ES cells in the host myocardium, TUNEL staining was performed (Figure 14A-L). At D14, there was a significant decrease in TUNEL-positive nuclei post-MI in the infarct and peri-infarct regions of hearts transplanted with ES cells compared to controls (Figure 14Q, $p<0.001$). Moreover, TIMP-1-ES cell transplanted hearts contained significantly fewer total apoptotic nuclei compared to ES cell and cell culture medium groups (mean ± SE; MI + TIMP-1-ES cells: 0.63 ± 0.06% vs. MI + ES cells: 0.94 ± 0.07% and MI + CC: 1.55 ± 0.12%, TUNEL positive/total nuclei, $p<0.05$, Figure 14Q). Additionally, heart sections were colabeled with sarcomeric α-actin to demonstrate and quantitate apoptosis within cardiac myocyte nuclei (Figure 14M-P).

Our data shows apoptotic cardiac myocyte nuclei were significantly reduced in hearts transplanted with TIMP-1-ES cells compared with ES cell and media transplanted hearts (p<0.05, Figure 14R). Next, to strengthen our TUNEL data, a caspase-3 colorimetric assay was performed on heart homogenates from each group. There was a significant decrease in caspase-3 activity in hearts treated with TIMP-1-ES cells post-MI compared to hearts transplanted with ES cells or medium alone (mean ± SE; MI + TIMP-1-ES cells: 171.75 ± 2.56 vs. MI + ES cells: 188.50 ± 1.80 and MI + CC: 203.00 ± 4.36, p<0.05, Figure 14S).

TIMP-1 Enhances p-Akt Activation in Post-MI Hearts

Previous studies have shown that mechanisms of inhibited apoptosis involve the activation of cell-survival pathways including p-Akt (27). Our data demonstrates that hearts transplanted with TIMP-1-ES cells did in fact have significant increase in the activation of p-Akt compared to hearts transplanted with ES cells and medium (mean ± SE; MI + TIMP-1-ES cells: 6.99 ± 0.22, vs. MI + ES cells: 5.75 ± 0.16 and MI + CC 4.61 ± 0.25, p<0.05, Figure 15), suggesting up-regulation of cell survival pathways.

Reduced Fibrosis Following MI in TIMP-1-ES Cell Group

To quantify the amount of cardiac fibrosis, heart sections were stained with Masson's trichrome. Interstitial fibrosis in the infarcted heart sections was measured at D14 and representative photomicrographs from each group are depicted in Figure 16A-D. Compared to medium alone, ES cell transplanted hearts had a significant reduction in the amount of total interstitial fibrosis (Figure 16, right panel histogram, p<0.01). Furthermore, the reduced interstitial fibrosis observed in TIMP-1-ES cell transplanted

hearts was significant when compared to ES cell and control hearts (mean ± SE; MI + TIMP-1-ES cell: 0.23 ± 0.03 mm^2 vs. MI + ES cells: 0.47 ± 0.02 mm^2 and MI +CC: 0.90 ± 0.00 mm^2, $p<0.01$, Figure 16E).

To determine whether inhibition of fibrosis was associated with changes in MMP-9, a well-documented MMP involved in the pathogenesis of MI, MMP-9 was quantified using an enzyme-linked immunoassay. Hearts transplanted with ES cells demonstrated significantly reduced MMP-9 concentration compared with control hearts ($p<0.01$, Figure 16F). Moreover, MMP-9 concentration within TIMP-1-ES cell transplanted hearts was significantly less compared to hearts transplanted with ES cells or media (mean ± SE; MI + TIMP-1-ES cells: 21.19 ± 1.27 vs. MI + ES cells: 41.64 ± 3.83 and MI + CC: 63.81 ± 8.18, $p<0.01$, Figure 16F).

Next, quantitative assessment of vascular fibrosis (Figure 17A-D) revealed ES cell transplanted hearts had a significant decrease in the amount of vascular fibrosis compared to medium alone ($p<0.001$, Figure 17, right panel histogram). Furthermore, the attenuation of vascular fibrosis in TIMP-1-ES cell transplanted hearts reached statistical significance when compared to ES cells ($p<0.05$, Figure 17, right panel histogram).

Overexpression of TIMP-1 Improves Cardiac Function Post-MI

Cardiac function data assessed by echocardiography at D14 suggests mice receiving ES cell transplantation following MI had significantly improved fractional shortening compared to medium alone ($p<0.001$, Figure 18A). Mice transplanted with TIMP-1-ES cells post-MI had significantly increased cardiac function when compared to

mice transplanted with ES cells and medium post-MI at D14 (mean ± SE; MI + TIMP-1-ES cells: 44.81 ± 0.70% vs. MI +ES cells: 41.01 ± 0.61% and MI + CC: 31.30 ± 0.88%, $p<0.05$, Figure 18A). Furthermore, our ejection fraction data demonstrates mice transplanted with TIMP-1-ES cells had significantly enhanced ejection fraction when compared with ES cell or control groups ($p<0.05$, Figure 18B).

Discussion

Many stem cell types examined thus far including skeletal myoblasts, bone marrow stem cells, bone marrow-derived hematopoietic stem cells, endogenous cardiac stem cells, mesenchymal stem cells, induced pluripotent stem (iPS) cells, and ES cells have shown improved cardiac function following transplantation post-MI(11; 19-22). Improvement in cardiac function in past studies was demonstrated by newly differentiated cardiac myocytes and inhibition of apoptosis and fibrosis. However, observed effects of stem cell transplantation following MI was not enough to provide complete protection required to reestablish normal heart function. Therefore new strategies, such as modulation of ES cells using anti-apoptotic and anti-fibrotic genes, are required. In the present study, we transfected ES cells with TIMP-1, an anti-apoptotic and anti-fibrotic gene, transplanted them into the myocardium post-MI, and evaluated their effects on myocardial remodeling (apoptosis and fibrosis) and cardiac function.

Apoptosis is a major mechanism by which cardiac myocyte cell death occurs following MI leading to hypertrophy, fibrosis and ultimately, poor cardiac function. TIMP-1, an intrinsic cardiac glycoprotein which plays a role in MMP inhibition and

mitogenic modulation, has recently been identified as anti-apoptotic in various cell lines including human osteosarcoma cells (MG-63), mouse bone marrow stromal cells (MBA-1), murine MC3T3-E1 osteoblasts, human breast epithelial cells, and rat cardiomyoblasts (H9c2)(17; 24; 30; 31). The present study demonstrates a significant reduction in apoptosis in the host myocardium following transplantation with TIMP-1-ES cells compared to ES cells confirmed by TUNEL staining and caspase-3 activity assay. Our data is in accordance with a previously published study suggesting TIMP-1 conditioned media (CM) prevents H_2O_2-induced apoptosis in an *in vitro* model similar to ischemic myocardium(24).

Previous studies have reported a positive correlation between TIMP-1 and activation of the Akt signaling cascade promoting breast epithelial and UT-7 erythroid cell survival(1; 14). Our present study also provides evidence indicating hearts transplanted with TIMP-1-ES cells had significant activation of p-Akt compared to hearts transplanted with ES cells and medium controls. Although future detailed studies are necessitated to determine the exact mechanisms of the TIMP-1 anti-apoptotic influence, our findings are in corroboration with previous studies suggesting mechanisms of inhibited apoptosis involve the activation of cell-survival pathways including Akt(27).

Fibrosis, a major contributor to adverse cardiac remodeling, is consequent to increased MMP activation post-MI which leads to ECM degradation, increased collagen deposition, stiffening of the heart, and ultimately, poor cardiac function. In the present study, our cardiac fibrosis data (interstitial and vascular fibrosis) demonstrates significantly reduced fibrosis in hearts treated with TIMP-1-ES cells compared with hearts transplanted with ES cells. Additionally, hearts transplanted with TIMP-1-ES

cells contained significantly less MMP-9 compared with ES cell and CC groups post-MI. Following an MI, increased MMP activation is not paralleled by an increase in TIMP-1 expression(32). Therefore, we suggest significant reduction in the post-MI fibrosis observed in the TIMP-1-ES cell transplanted hearts compared to ES cell transplanted hearts may be attributable to inhibition of MMP-9 through overexpression of TIMP-1. However, further studies are required to define these mechanisms.

Finally, we needed to determine whether inhibition of apoptosis and fibrosis in the host myocardium contribute to improved cardiac function following MI in TIMP-1-ES cell transplanted animals. In the present study, our data demonstrates that 2 weeks following MI, mice transplanted with TIMP-1-ES cells had significantly increased fractional shortening and ejection fraction compared to mice transplanted with ES cells or cell culture medium. Overall, our findings indicate that transplantation of TIMP-1-ES cells attenuate adverse cardiac remodeling and improve heart function following MI.

In conclusion, the major findings of the present study relative to ES cells include; 1) TIMP-1-ES cells significantly inhibit cardiac apoptosis in the infarcted myocardium, 2) inhibition of apoptosis in hearts transplanted with TIMP-1-ES cells is mediated, in part, through the Akt pathway, 3) transplanted TIMP-1-ES cells significantly inhibit interstitial and vascular fibrosis as well as the pro-fibrotic protein, MMP-9, following MI, and 4) transplanted TIMP-1-ES cells significantly improve overall cardiac function following MI through retention of functional cardiac myocytes and maintenance of the integrity of the host myocardium. However, future studies are required to identify further detailed mechanisms by which TIMP-1 prevents apoptosis and fibrosis in the infarcted myocardium.

Funding

This work was supported, in part, from grants from the National Institutes of Health [1R01HL090646-01, and 5R01HL094467-02 to DKS].

Acknowledgements

The authors would like to thank Dr. Xilin Long for the generation of TIMP-1-ES cells, Reetu Singla for ES cell culture maintenance, Dr. Binbin Yan for assistance with confocal images, and Ajitha Dammalapati for histological assistance.

Figures

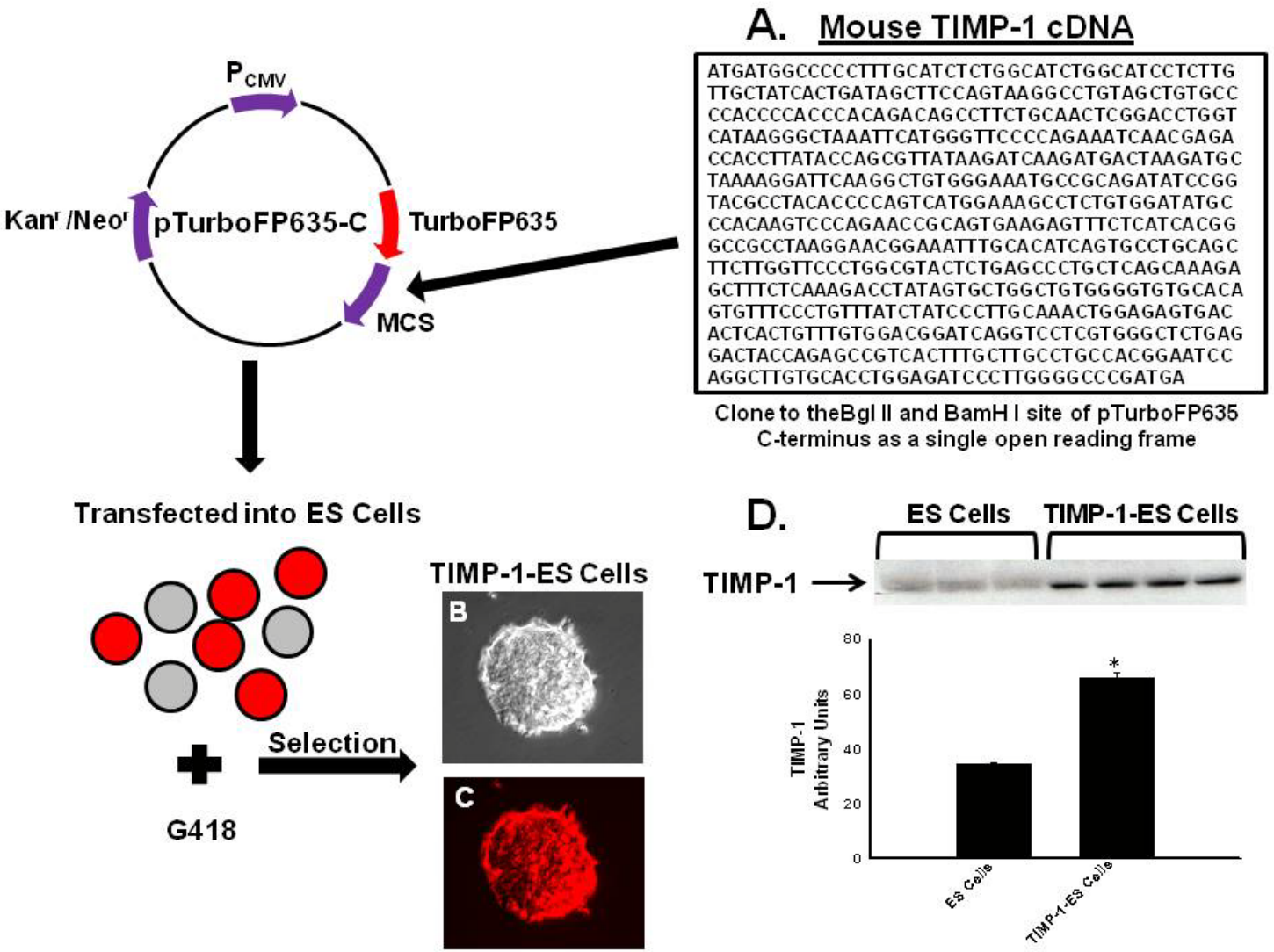
A. Mouse TIMP-1 cDNA
ATGATGGCCCCCTTTGCATCTCTGGCATCTGGCATCCTCTTG
TTGCTATCACTGATAGCTTCCAGTAAGGCCTGTAGCTGTGCC
CCACCCCACCCACAGACAGCCTTCTGCAACTCGGACCTGGT
CATAAGGGCTAAATTCATGGGTTCCCCAGAAATCAACGAGA
CCACCTTATACCAGCGTTATAAGATCAAGATGACTAAGATGC
TAAAAGGATTCAAGGCTGTGGGAAATGCCGCAGATATCCGG
TACGCCTACACCCCAGTCATGGAAAGCCTCTGTGGATATGC
CCACAAGTCCCAGAACCGCAGTGAAGAGTTTCTCATCACGG
GCCGCCTAAGGAACGGAAATTTGCACATCAGTGCCTGCAGC
TTCTTGGTTCCCTGGCGTACTCTGAGCCCTGCTCAGCAAAGA
GCTTTCTCAAAGACCTATAGTGCTGGCTGTGGGGTGTGCACA
GTGTTTCCCTGTTTATCTATCCCTTGCAAACTGGAGAGTGAC
ACTCACTGTTTGTGGACGGATCAGGTCCTCGTGGGCTCTGAG
GACTACCAGAGCCGTCACTTTGCTTGCCTGCCACGGAATCC
AGGCTTGTGCACCTGGAGATCCCTTGGGGCCCGATGA
Clone to theBgl II and BamH I site of pTurboFP635
C-terminus as a single open reading frame
P CMV
Kanr /Neor
pTurboFP635-C
TurboFP635
MCS
Transfected into ES Cells
G418
Selection
TIMP-1-ES Cells
B
C
D.
ES Cells
TIMP-1-ES Cells
TIMP-1
TIMP-1 Arbitrary Units
80
60
40
20
0
*
ES Cells
TIMP-1-ES Cells

Figure 13. TIMP-1 vector and the generation of TIMP-1-ES cells. A: TIMP-1 cDNA was cloned into the pTurboFP635-C expression vector encoding RFP. TIMP-1-ES cells were selected following incubation with G418. Representative photomicrographs of TIMP-1-ES cells in bright field microscopy (B) and under fluorescence microscopy (C) to demonstrate RFP expression. D: Representative photomicrographs of western blot. Histogram shows significant overexpression of TIMP-1 in TIMP-1-ES cells relative to parental ES cells. *$p<0.001$ vs. ES cells.

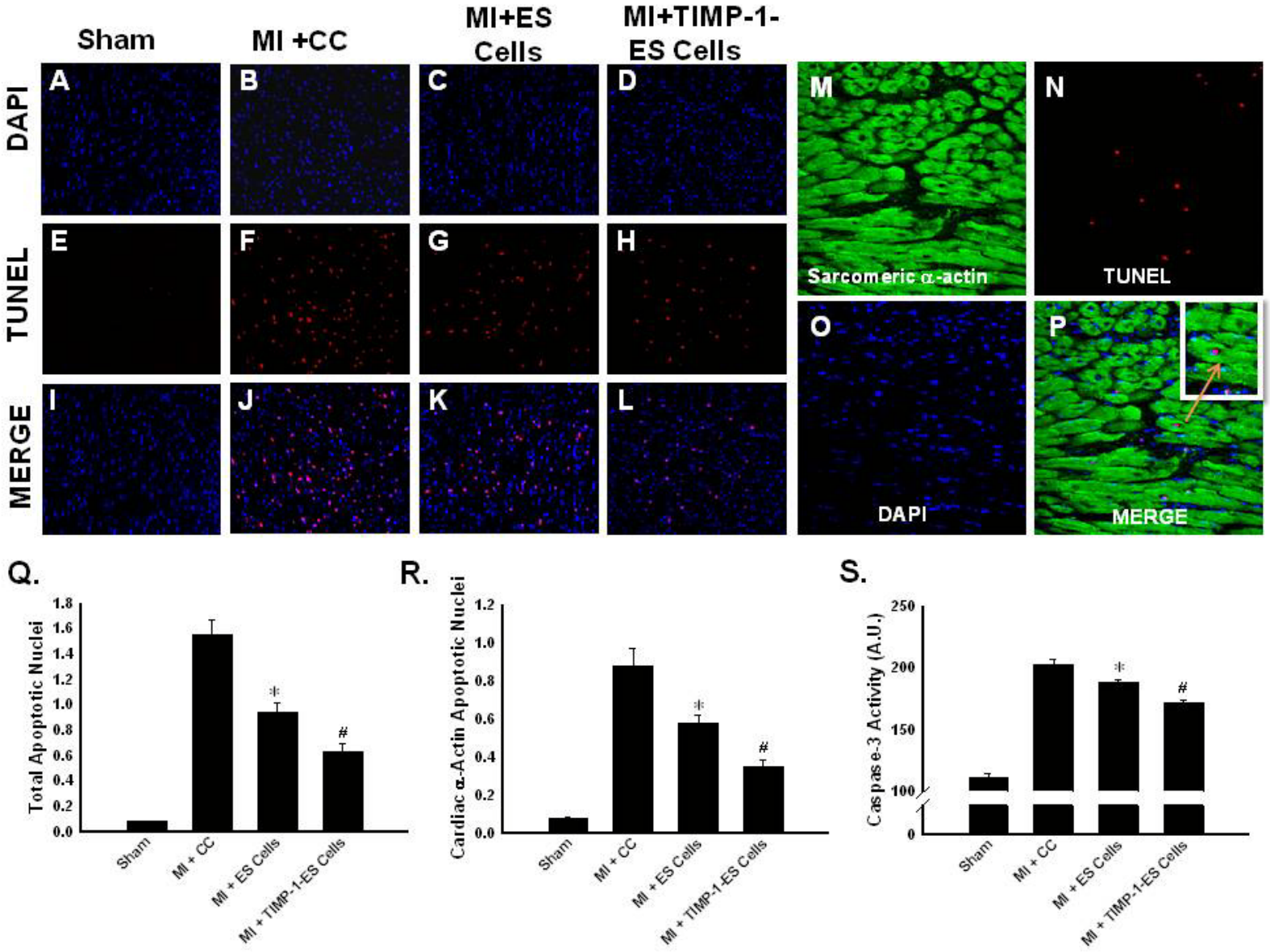
Sham
MI +CC
MI+ES Cells
MI+TIMP-1-ES Cells
DAPI
TUNEL
MERGE
A
B
C
D
E
F
G
H
I
J
K
L
M
Sarcomeric α-actin
N
TUNEL
O
DAPI
P
MERGE
Q.
Total Apoptotic Nuclei
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells
R.
Cardiac α-Actin Apoptotic Nuclei
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells
S.
Caspase-3 Activity (A.U.)
250
200
150
100
0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells

Figure 14. Effects of transplanted ES cells overexpressing TIMP-1 on host cardiac myocyte apoptosis in C57BL/6 mice. ES and TIMP-1-ES cells were transplanted post-MI and hearts were examined for apoptosis 2 wks following cell transplantation. Representative photomicrographs of total nuclei stained with DAPI in blue (A-D), apoptotic nuclei stained with TUNEL in red (E-H), and merged nuclei in pink (I-L). Magnification, 40X. Top right panel (M-P), set of representative photomicrographs of section labeled with sarcomeric α-actin (M), apoptotic nuclei stained with TUNEL in red (N), total nuclei stained with DAPI in blue (O), and merged image (P) demonstrating apoptosis occurs within the cardiac myocyte. Magnification, 40X. Boxed area in panel O enlarged to show colocalization of sarcomeric α-actin, TUNEL positive nucleus, and DAPI. Bottom left histogram (Q) shows total apoptotic nuclei in the infarct and peri-infarct regions of the heart in 1-2 sections from 5-8 hearts from each group. *p<0.001 vs. MI + CC, #p <0.05 vs. MI + CC and MI + ES cells. Bottom middle histogram (R) shows quantitative cardiac myocyte apoptotic data. *p<0.01 vs. MI + CC, #p<0.05 vs. MI + CC and MI + ES cells. Bottom right histogram (S) shows quantitative analysis of caspase-3 activity in hearts transplanted with and without stem cells. *p<0.05 vs. MI + CC, #p<0.05 vs. MI + CC and MI + ES cells. Data set are from heart homogenates of n=8 animals/group. A.U. = arbitrary units.

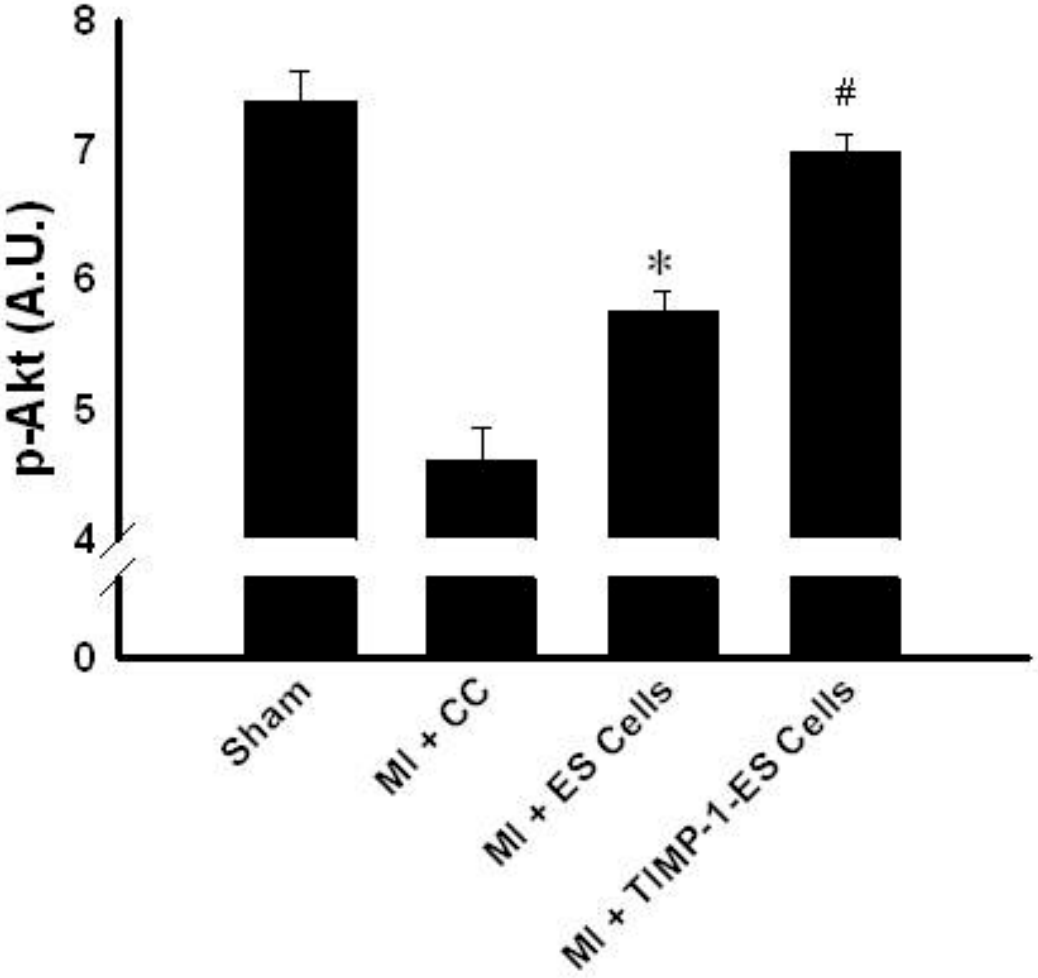
8
7
6
5
4
0
p-Akt (A.U.)
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells

Figure 15. Phospho-Akt activation enhanced in TIMP-1-ES cell transplanted hearts. Histogram shows quantitative analysis of phospho-Akt activation. *p<0.01 vs. MI + CC, #p<0.01 vs. MI + CC and MI + ES cells. Data set are from heart homogenates of n=8 animals/group. A.U. = arbitrary units.

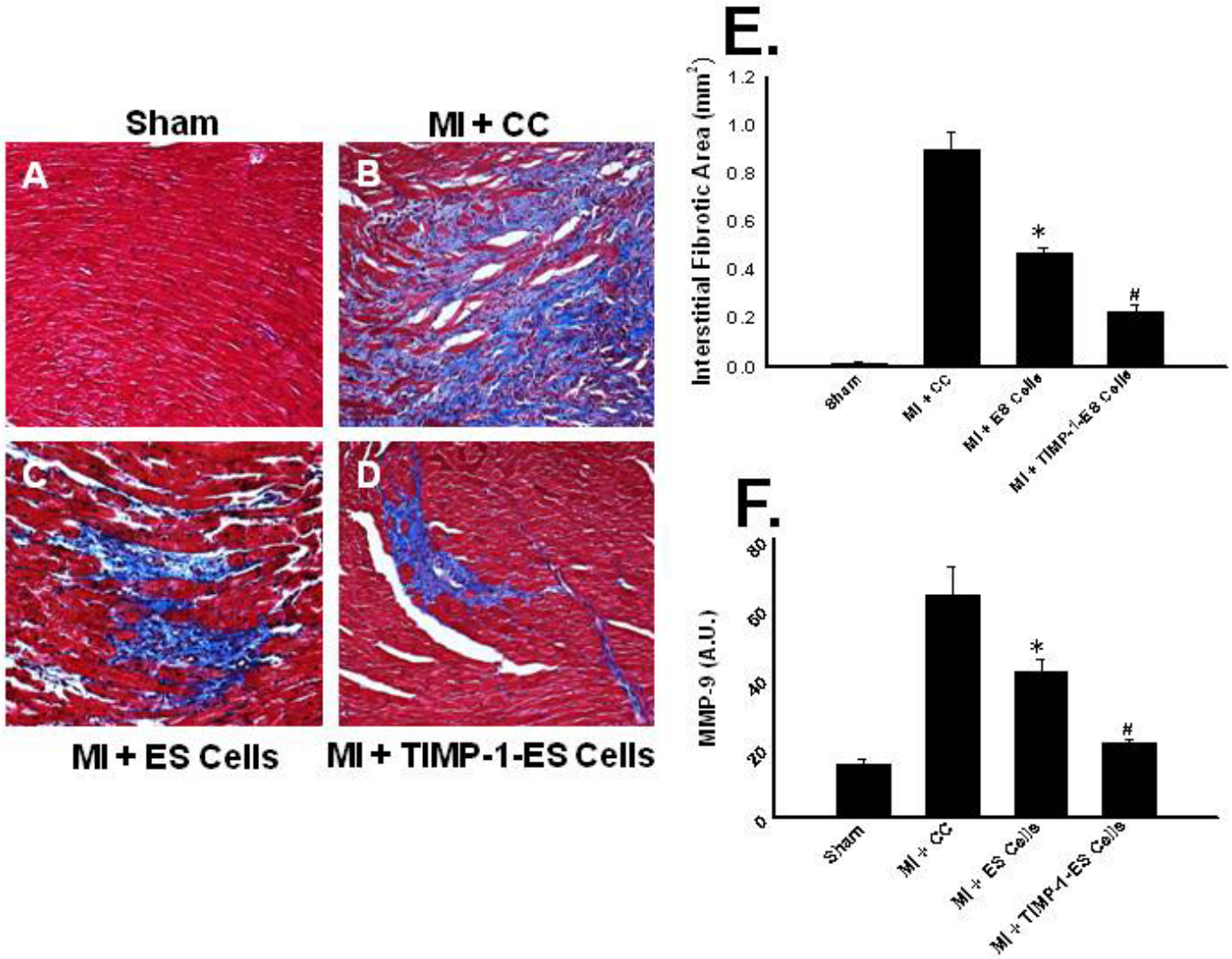
Sham
MI + CC
A
B
C
D
MI + ES Cells
MI + TIMP-1-ES Cells
E.
Interstitial Fibrotic Area (mm²)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells
F.
MMP-9 (A.U.)
80
60
40
20
0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells

Figure 16. Effects of transplanted ES cells overexpressing TIMP-1 on interstitial fibrosis. Representative photomicrographs from sections stained with Masson's trichrome 2 wks following coronary artery ligation from each group (A-D). Magnification, 20X. E: Histogram shows quantitative total interstitial fibrosis per section. **p*<0.01 vs. MI + CC, #*p*<0.05 vs. MI + CC and MI + ES cells. Data sets are from 1-2 sections from 5-8 hearts/group. F: Histogram shows average MMP-9 concentration within hearts from n=5-8 animals per group. **p*<0.01 vs. MI + CC, #*p*<0.05 vs. MI + CC and MI + ES cells. A.U. = arbitrary units.

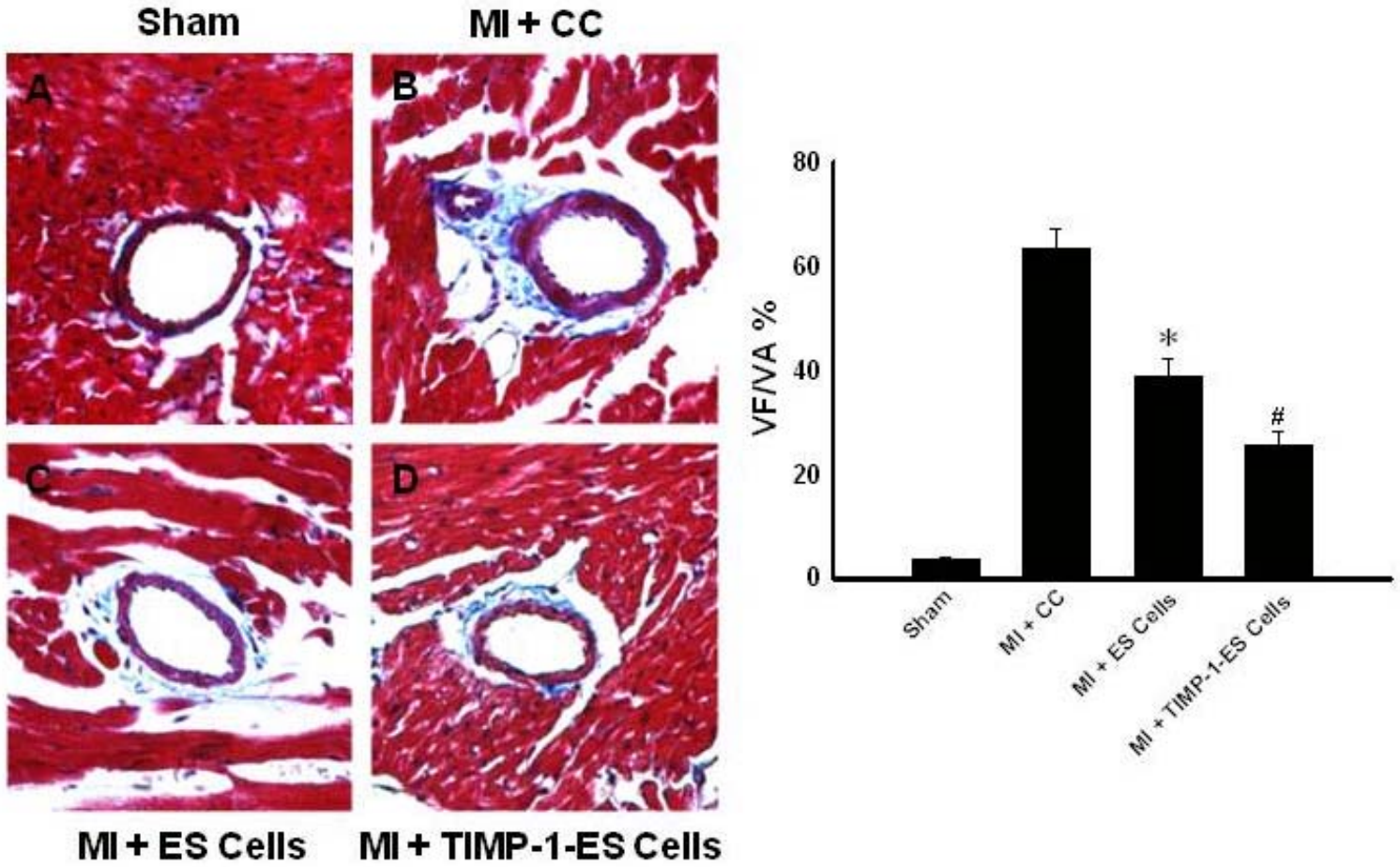
Sham
MI + CC
A
B
C
D
MI + ES Cells
MI + TIMP-1-ES Cells
VF/VA %
80
60
40
20
0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells

Figure 17. Transplanted TIMP-1-ES cells inhibit vascular fibrosis following MI. Representative photomicrographs of large vessels stained with Masson's trichrome 2 wks post-MI to demonstrate vascular fibrosis (A-D). Right panel: Histogram representing quantitative analysis of vascular fibrosis in heart sections. *p<0.001 vs. MI + CC, #p<0.01 vs. MI + CC and MI + ES cells. VA = vessel area, VF = vessel fibrosis. Data set are from 1-2 sections from 5-8 hearts/group.

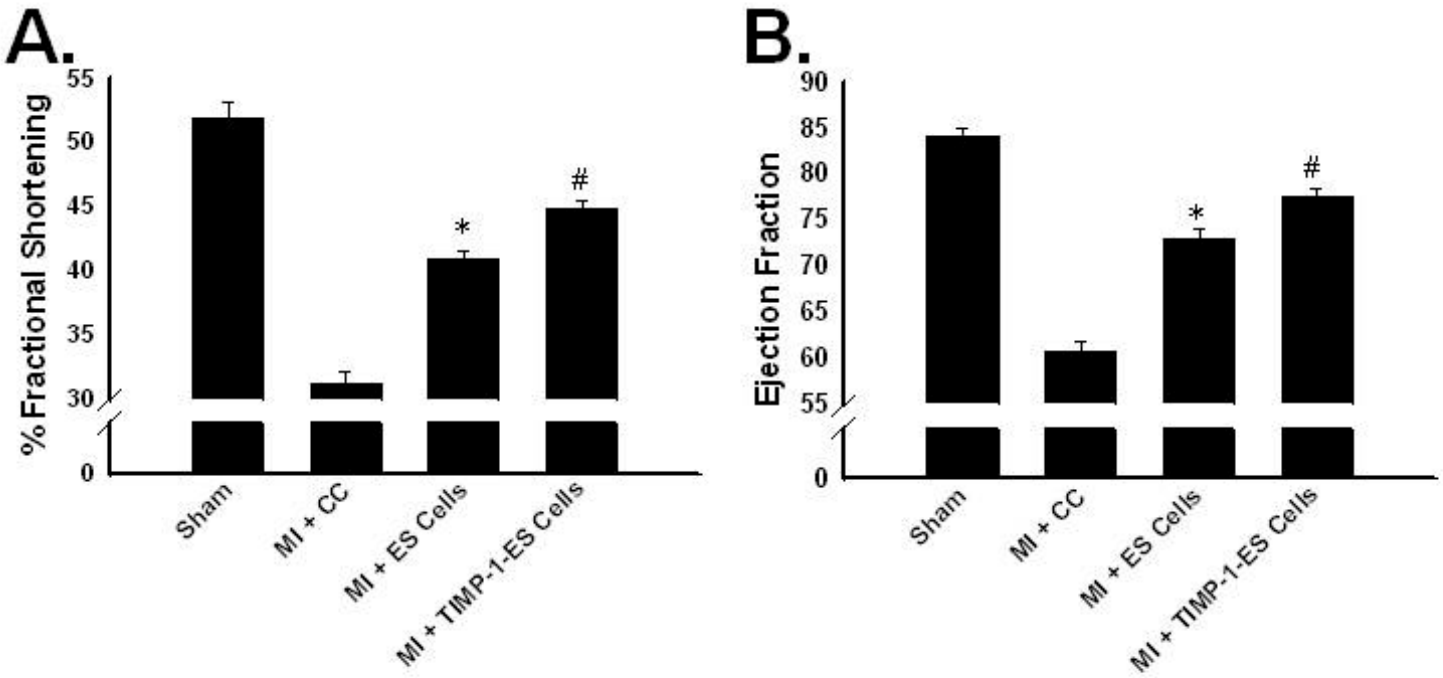
A.
% Fractional Shortening
55
50
45
40
35
30
0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells
B.
Ejection Fraction
90
85
80
75
70
65
60
55
0
*
#
Sham
MI + CC
MI + ES Cells
MI + TIMP-1-ES Cells

Figure 18. TIMP-1 overexpressed in ES cells improves cardiac function in C57BL/6 mice. Echocardiography was performed D14 following MI and fractional shortening and ejection fraction was quantified. A: Average fractional shortening at 2 wks post-MI for all treatment groups. *p<0.001 vs. MI + CC, #p<0.05 vs. MI + CC and MI + ES cells. FS=fractional shortening. B: Histogram shows quantified average ejection fraction. *p<0.001 vs. MI + CC, #p<0.05 vs. MI + CC and MI + ES cells. Data set are from n=8 different animals/group.

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CHAPTER FIVE: DISCUSSION

Although previous investigations have demonstrated inhibited cardiac remodeling and improved cardiac function following ES cell transplantation into experimental infarcted myocardium, results are modest(4; 7; 16; 23). The general consensus among researchers suggests refinement and optimization of stem cell populations are necessary to transcend stem cell therapy from concept to actuality for use in the clinical arena(28; 30). Tremendous efforts are now underway to enhance the efficacy by which ES cells differentiate into cardiac cell types following transplantation and inhibit apoptosis and fibrosis in the infarcted myocardium. In this regard, we generated ES cells overexpressing miR-1, a pro-cardiac microRNA, and TIMP-1, an anti-apoptotic and anti-fibrotic gene, transplanted them into the peri-infarct region post-MI, and evaluated their effects on engraftment, differentiation, and myocardial remodeling.

We suggest, for the first time, generated ES cells overexpressing miR-1, following transplantation into the infarcted myocardium, demonstrate dramatically increased donor cell-derived differentiated cardiac myocytes compared with transplanted, untransfected ES cells. Our *in vivo* data authenticates previous reports indicating miR-1 plays an integral role in early cardiac development in *Drosophila* and mice as well as mediates cardiac myocyte differentiation *in vitro*(13; 35; 36). Numerous studies have identified specific miR-1 targets involved in early cardiac organogenesis, including histone deacetylase 4 (HDAC4), heart and neural crest derivatives-expressed protein 2 (Hand2), notch ligand delta-like 1 (Dll-1) and cyclin-dependent kinase-9 (Cdk9) (5; 6; 12; 26). To understand and delineate mechanism by which miR-1 drives cardiac

myocyte differentiation from transplanted ES cells in the infarcted myocardium will require future investigation.

Next, it was pertinent to investigate whether transplanted miR-1-ES cells promote additional repair mechanisms (apoptosis and fibrosis inhibition) in the injured myocardium. In the first study (Chapter 2), we report that inhibition of apoptotic cell death is significantly different in miR-1-ES and ES cell transplanted hearts compared to cell culture medium controls. Notably, we demonstrate a significant reduction in intrinsic cardiac myocyte apoptotic cell death in miR-1-ES cell transplanted hearts compared to hearts transplanted with ES cells. Our data is the first report suggesting endogenous cardiac myocyte apoptosis inhibition is mediated by miR-1. Conversely, previous studies have concluded miR-1 mediates apoptosis in cardiac myocytes by post-transcriptional repression of HSP60, HSP70, caspase-9, IGF-1 and Bcl2(22; 27; 33). However, the experimental design in our study (miR-1 expression in ES cells) and the studies by which miR-1 is pro-apoptotic (miR-1 expression in cardiac myocytes) are vastly unique which may explain the discrepancies between these studies. Previous research has concluded that miR-1 expression increases in neonatal hearts and substantially abundant levels are maintained in the adult heart(33). However, miR-1 is not abundantly expressed in ES cells as we have demonstrated with our RT-PCR data. Therefore we suggest that the behavior of miR-1 within ES cells (anti-apoptotic) may be uniquely different than that in cardiac myocytes (pro-apoptotic) by targeting different mRNAs found in each cell type. Indeed, upon a review of published miR-21 data, Wang et al suggested that the behavior of a miR can and does vary based on the cell line in which it is being expressed(31).

To elucidate mechanisms of miR-1-mediated apoptosis inhibition in the infarcted myocardium, we investigated the relationship of miR-1 and the PI3K/Akt pathway. The PI3K/Akt signaling pathway is involved in a host of biological activities including cell survival, growth, and proliferation. Phosphorylated and activated Akt promotes cell survival by phosphorylating and activating downstream anti-apoptotic substrates and by phosphorylating and inactivating pro-apoptotic substrates. Akt has been shown to be a key player promoting cardioprotection and anti-apoptotic regulation in the ischemic myocardium(10). More so, previous studies have shown an up-regulation of activated p-Akt following cell transplantation in the post-MI heart(25). We assessed the levels of activated Akt and revealed a positive correlation between p-Akt and apoptosis inhibition. Notably, as apoptosis was dramatically decreased in miR-1-ES cell transplanted hearts, p-Akt expression was significantly increased compared with ES cell and CC transplanted hearts. Importantly, we suggest that the decreased adverse remodeling observed within our study using transplanted miR-1-ES cells is *partially* consequent to increased p-Akt expression. Previous studies have shown that Akt-mediated paracrine factors including secreted frizzled related protein 2 and follistatin-like 1 promote survival and repair of the injured myocardium(17; 19). We suggest that increased Akt expression observed within the miR-1-ES cell transplanted hearts leads to the observed cardioprotective effects through increased secreted paracrine factors. Using miR mRNA target prediction websites (targetscan.org and microrna.org), we were able to conclude that Akt mRNA is not a predicted miR-1 target as we did not expect it to be. To that end, we turned our attention to upstream modulators of the PI3K/Akt pathway to identify a link between miR-1 and up-regulated activated Akt.

PTEN inhibits the PI3K/Akt pathway by dephosphorylating PIP_3 and subsequently blocking the phosphorylation and activation of Akt. We hypothesized that down-regulation of PTEN would lead to increased levels of p-Akt as observed within our study. We assessed levels of PTEN and were able to conclude that levels of PTEN were in fact significantly decreased in hearts transplanted with miR-1-ES cells compared with respective controls. Using bioinformatics, we were also able to extrapolate miR-1 does not directly target PTEN mRNA. However, we hypothesize that miR-1 may down regulate activators of PTEN thus leading to increased activated Akt expression within miR-1-ES cell transplanted hearts. In fact, there was a recent publication demonstrating a mechanistic relationship between miR-101 and activation of Akt through down regulation of a PTEN activator as we suggest within our study for miR-1(21). However, because miR-1 is not limited to single target regulation, determination of the miR-1 target mRNAs responsible for the inhibition of apoptosis is well beyond the scope of Study 1 and will require further investigation.

As stated previously, the vast predicted mRNA targets for miR-1 span a host of biological activities. Therefore, we examined whether miR-1 could also confer cardioprotection to the injured myocardium through inhibition of oxidative stress. Oxidative stress has been shown to play a key role in myocardial apoptosis and subsequent adverse remodeling following MI(2; 24). We were able to demonstrate that transplantation of miR-1-ES cells following MI significantly inhibited superoxide anion production. Our study suggests that miR-1-ES cells protect the host myocardium from MI-induced apoptosis not only through activation of the Akt pathway, but also through inhibition of oxidative stress.

In other respects, we investigated the role of transplanted miR-1-ES cells on fibrosis formation post-MI as adverse remodeling involves the production of excess fibrous connective proteins to rescue the infarcted ventricular geometry. Interstitial and vascular fibrosis was dramatically decreased in hearts transplanted with ES and miR-1-ES cells compared with controls. However, statistical significance was not attained in fibrosis inhibition between ES and miR-1-ES cell groups. MMP-9, a mediator of fibrosis in the infarcted heart, was also quantified to verify our fibrosis results. Our data demonstrates that although significant to cell culture medium controls, there was no significant reduction in MMP-9 expression in hearts transplanted with ES and miR-1-ES cells supporting our quantitative interstitial and vascular fibrosis data.

Finally, in Study 1, we needed to determine whether enhanced cardiac myocyte differentiation of transplanted miR-ES cells and inhibited apoptosis contributed to improved cardiac function as the success of stem cell transplantation ultimately is defined by the improvement in left ventricular contractility and output. Previous studies have revealed improved cardiac function following transplantation of various stem cell populations in the infarcted heart(9; 16; 18; 23). We demonstrate that 2 weeks following MI, fractional shortening and ejection fraction are significantly improved in miR-1-ES cell transplanted mice compared to ES cell transplanted mice. Conceivably, we suggest that enhanced cardiac myocyte differentiation from transplanted miR-1-ES cells and inhibition of myocardial apoptosis contribute to the improved left ventricular function.

Study 2 (Chapter 3) was undertaken to assess the role of miR-1 and apoptosis inhibition in a chronic model of MI. Apoptosis accounts for millions of cardiac myocytes lost following MI(2). Research has shown that apoptosis not only occurs in hours but

also months following ischemia suggesting apoptosis plays a role in remodeling and the development of heart failure(1). Given this, the challenge to generate effective therapies which will protect the host myocardium from apoptosis at short and long term following MI remains ever present. In Study 1, we were able to demonstrate the anti-apoptotic effects of miR-1 overexpressing ES cells following transplantation into the injured myocardium in an acute setting of MI. Whether miR-1 overexpressing ES cells following transplantation in the infarcted myocardium could inhibit apoptosis 4 weeks post-MI was unknown.

Evidences provided in Study 2 demonstrate a novel role of miR-1 in the regulation of host myocardium apoptosis 4 weeks following MI. We reveal that apoptosis, assessed by TUNEL staining and a caspase-3 activity assay, was significantly reduced in hearts transplanted with miR-1-ES cells compared with controls suggesting the anti-apoptotic effects of miR-1 were long lasting. We further assessed whether mechanisms by which miR-1 obviates apoptosis in the acute model of MI were similar to those at 4 weeks post-MI. As observed in Study 1, levels of p-Akt were significantly improved in the miR-1 group compared with the ES cell and cell culture media groups. We also quantified levels of PTEN and revealed that at 4 weeks post-MI, PTEN expression was dramatically decreased compared with respective controls. Our data suggest that mechanisms of inhibited apoptosis by miR-1-ES cells in the chronic model of MI are similar to those in an acute model of MI.

Finally, we wanted to investigate the impact of apoptosis inhibition by miR-1-ES cells on cardiac function at 4 weeks following MI. *In vivo* delivery of miR-1-ES cells significantly improved fractional shortening and ejection fraction 4 weeks following

transplantation and MI. Our data is in corroboration with a previously published study demonstrating *in vivo* delivery of miR-24 suppressed apoptosis in the injured myocardium leading to improved contractile function(20). Our data from Study 2 suggests that miR-1, when overexpressed in transplanted ES cells, has the capacity to inhibit apoptosis long term while attenuating contractility loss.

In Study 1, the goal was to enhance cardiac myocyte differentiation from transplanted ES cells using miR-1 which addresses one of the major issues of current stem cell therapy. In an attempt to address the issue of limited adverse remodeling inhibition, we overexpressed TIMP-1, an intrinsic cardiac glycoprotein, in ES cells in Study 3 (Chapter 4). TIMP-1, an anti-fibrotic protein which modulates MMP activation and mitogenic regulation, has recently been shown to exhibit anti-apoptotic characteristics in various cell lines including human osteosarcoma cells, mouse bone marrow stromal cells, murine osteoblasts, human breast epithelial cells, and rat cardiomyoblasts(15; 24; 29; 32). In Study 3, we were able to show that myocardial apoptosis was significantly decreased in hearts transplanted with TIMP-1-ES cells compared to hearts transplanted with ES cells or cell culture media. Our data corroborates our previously published *in vitro* data suggesting TIMP-1 inhibits H_2O_2 induced apoptosis in cardiomyoblasts.

Along the same line of thought in Study 1, we wanted to see if a relationship existed between transplantation of TIMP-1-ES cells and activation of the PI3K/Akt pathway to explain the significant inhibition of apoptosis. Previous studies in breast epithelial and erythroid cells had demonstrated an association between TIMP-1 and cell survival through activation of the PI3K/Akt pathway(3; 14). Our data suggests p-Akt

expression was significantly increased in hearts transplanted with TIMP-1-ES cells compared with ES and cell culture medium controls. Although the precise mechanism detailing the role of TIMP-1 in apoptosis inhibition has not been elucidated, we suggest TIMP-1 perpetuates its anti-apoptotic influence through activation of the PI3K/Akt pathway.

Fibrosis, a chief component of adverse cardiac remodeling, involves increased MMP activation post-MI which leads to ECM degradation, increased collagen deposition, stiffening of the heart, and ultimately, poor cardiac function. TIMP-1 is well established as an endogenous inhibitor of MMPs by binding the active site of secreted and membrane bound pro-MMPs preventing cleavage of the pro-peptide domain and subsequent activation(8; 11). However, following an MI, increased MMP activation is not paralleled with increased TIMP-1 expression(34). Therefore, we hypothesized that TIMP-1when overexpressed in transplanted ES cells in the post-MI heart would significantly inhibit cardiac fibrosis through inhibition of MMP activation. Our data substantiates our hypothesis as interstitial and vascular fibrosis was significantly decreased in hearts transplanted with TIMP-1-ES cells compared to large fibrotic areas in cell culture media transplanted hearts. Additionally, expression levels of MMP-9, a pivotal MMP in cardiac fibrosis post-MI, were significantly decreased in TIMP-1-ES cell groups compared with ES cell and cell culture medium controls. We suggest that TIMP-1, when overexpressed in transplanted ES cells post-MI, exerts its anti-fibrotic influence through inhibition of MMP-9 activation and consequential ECM degradation and increased ECM protein production.

Finally, in Study 3, we wanted to determine if inhibition of maladaptive left ventricular remodeling by transplanted TIMP-1-ES cells sufficed to improve contractile function of the infarcted myocardium. Our functional data reveals that mice transplanted with TIMP-1-ES cells post-MI had significantly improved fractional shortening and ejection fraction compared to mice receiving ES cells or cell culture media. We suggest that the dramatic improvement in cardiac function in hearts transplanted with TIMP-1-ES cells is consequent to inhibition of host myocardial apoptosis and fibrosis.

Conclusions drawn from Study 1, 2, and 3 include: 1) miR-1 promotes cardiac myocyte differentiation of transplanted ES cells in the infarcted myocardium, 2) miR-1-ES cells suppress apoptosis in the acute setting of MI, 3) inhibited apoptosis mediated by miR-1-ES cells involves activation of Akt and down-regulation of PTEN, 4) reduced apoptosis following transplantation of miR-1-ES cells is also mediated though attenuation of ROS, 5) miR-1-ES cells inhibit interstitial and vascular fibrosis relative to control MI, 6) transplanted miR-1-ES cells improve left ventricular contractility at 2 weeks post-MI, 7) transplanted miR-1-ES cells suppress apoptosis in the chronic model of MI, 8) mechanism of apoptosis inhibition by miR-1-ES cells are similar in the chronic and acute model of MI, 9) miR-1-ES cell transplantation attenuates contractility loss at 4 weeks following MI, 10) TIMP-1-ES cells significantly inhibit host myocardium apoptosis post-MI, 11) apoptosis inhibition following TIMP-1-ES cell transplantation is mediated, in part, through the Akt pathway, 12) transplanted TIMP-1-ES cells inhibit interstitial and vascular fibrosis formation as well as activation of MMP-9 post-MI, and 13) transplanted TIMP-1-ES cells significantly improve overall cardiac function following MI through

retention of functional cardiac myocytes and maintenance of the integrity of the host myocardium. Our data provided in the 3 studies underscores the potential for imploring genetic manipulation of ES cells to generate an ideal stem cell population for use in the treatment of MI. Whether insertion of multiple microRNAs/transgenes such as miR-1and TIMP-1 into ES cells will generate a stem cell population capable of the combined beneficial effects observed with miR-1-ES cells and TIMP-1-ES cells independently remains to be seen. However, our research provides strong evidences of stem cell optimization to bring ES cell transplantation therapy from the bench to the clinic.

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